OCT 7 - 1931

THE AMERICAN JOURNAL OF PHYSIOLOGY

EDITED FOR

THE AMERICAN PHYSIOLOGICAL SOCIETY

Medical Lib.

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VOL. XCVIII—No. 3

Issued October 1, 1931

BALTIMORE, U. S. A.

1931

Entered as second-class matter, August 18, 1914, at the Post Office at Baltimore, Md., under the Act of March 3, 1879. Acceptance for mailing at special rate of postage provided for in section 1103, Act of October 3, 1917. Authorized on July 5, 1918

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THE AMERICAN
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VOL. 98 OCTOBER 1, 1931 No. 3

THE DETERMINATION OF THE AMOUNT OF BLOOD IN THE CENTRAL NERVOUS SYSTEM AFTER INJECTION OF HYPERTONIC SOLUTIONS

ARTHUR WEIL, F. R. ZEISS AND D. A. CLEVELAND

From the Institute of Neurology, Northwestern University Medical School, Chicago

Received for publication May 16, 1931

The facts which had been presented to support the different theories for the explanation of the effect of intravenous injections of hypertonic salt solutions on the central nervous system were based either on observations of the pressure of the cerebro-spinal fluid (Weed and McKibben, 1919; Weed and Hughson, 1921, and Ernst, 1930), the reaction of the meningeal blood-vessels (Wolff and Forbes, 1928) and the cerebral vessels (Kubie and Hetler, 1928) or studies of the blood-flow (Pilcher, 1930), combined with histologic analysis of the changes of the different nervous elements (Schaltenbrand and Bailey, 1927, and Sand, 1930).

The purpose of the present investigation was to determine directly the amount of blood which is present in the brain and spinal cord of the experimental animals under different conditions. Our present knowledge of the blood-content of the different organs of the body is mainly based on the experiments of Johannes Ranke (1871). He perfused the different organs after their removal with sodium chloride solution and determined the hemoglobin content of the fluid. Our own method was based on the observation of Voigt (1914) that brain and spinal cord do not contain colloidal silver four hours after the intravenous injection of collargol. Or, in other words, if one determines the amount of silver which is found in the brain a short time after the intravenous injection of collargol one may be sure that all the silver was circulating in the blood of the brain, but had not been absorbed by this organ itself. For other organs the conditions are different; the liver, e.g., takes more than 30 per cent of the silver, which had been injected, very rapidly out of the circulation.

Collargol (Heyden) solutions of 4.4 per cent in either 0.85 per cent or 15 per cent sodium chloride solutions were prepared, using corn syrup

(5 per cent) as a protective colloid in order to prevent precipitation of the colloidal silver. After a given time (5 to 15 minutes following the injection being the interval for obtaining maximal reduction of the spinal fluid pressure in Weed's experiments) 20 to 50 cc. of blood were collected from the femoral artery and mixed with a few milligrams of heparin. The animal was killed immediately thereafter by puncture of the medulla oblongata; both carotid arteries and jugular veins were ligated, the skull was opened, the medulla oblongata was ligated "en masse" and the brain was removed. It was dried for the determination of water and ashed with sulfuric and nitric acids. In the acid solution silver was determined with the Volhard method using m/50 solutions of ammonium-sulfocyanide

TABLE 1

Injected 4.37 per cent collargol in 0.85 per cent NaCl, 8 cc. per kgm.

NUMBER	RACE	WEIGHT OF		BRAIN IN PER CENT OF BODY	BLOOD IN BRAIN			PER CENT WATER IN	
		Body	Brain		cc.	Per 100 grams	Per cent of total	Brain	Blood
		kgm.	*grams*						
1	Spitz	6.1	52.9	0.87	3.7	7.1	1.1	77.9	
2	Spitz	6.8	62.9	0.93	3.9	6.2	0.95	77.8	79.5
3	Poodle	7.3	66.0	0.90	3.9	5.6	0.88	78.6	78.5
4	Spitz	8.2	59.3	0.72	4.1	6.9	0.83	72.5	
5	Dachs	8.6	59.4	0.69	5.5	9.2	1.0	76.1	77.8
6	Spitz	9.2	56.7	0.61	6.2	10.9	1.1	78.5	
7	Collie	13.6	78.8	0.58	5.0	6.4	0.61	76.5	80.0
8	Police	17.5	82.0	0.47	9.6	11.7	0.90	79.4	79.5
9	Collie	25.4	77.7	0.31	7.3	9.1	0.50	76.9	74.7
Average.....		11.4	66.1	0.58	5.5	8.3	0.79*	77.2	78.3

* The total amount of blood present was calculated by using the average amount of 6.6 per cent of the body weight as given by Ranke.

for the titration. At the same time a silver determination was carried out in an ashed amount of 30 to 50 cc. of blood. From the amount of silver which was found in 1 cc. of blood and in the whole brain the amount of blood was calculated which had been contained in the brain.

Tables 1, 2 and 3 give the results of the different experiments.

The relative weight of the brain expressed in percentage of body weight varies considerably. By choosing pairs of dogs of the same weight and arranging them in such a way that the average body weight is the same in both groups of experiments, comparisons are permissible. Our figures are an addition to the scanty material published in the literature. Hrdlička's (1905) figures, e.g., vary from 1.2 per cent of body weight in a dog of 5.7 kgm. to 0.69 per cent in a dog of 13.1 kgm. If one plots the brain weight of

terriers and collies on the abscissa of millimeter paper and the body weight on the ordinate, the different points of the resulting curve are fairly evenly distributed above and below a straight line connecting the lowest and highest points. If one applies the same method to brain weight and body weight of albino rats (Donaldson, 1915) one will find that the resulting

TABLE 2

Injected 4.37 per cent collargol in 15 per cent NaCl, 8 cc. per kgm.

NUMBER	RACE	WEIGHT OF		BRAIN IN PER CENT OF BODY	BLOOD IN BRAIN			PER CENT WATER IN	
		Body	Brain		cc.	Per 100 grams	Per cent of total	Brain	Blood
		kgm.	*grams*						
1	Collie	6.4	49.4	0.77	5.9	12.1	1.5	78.1	
2	Dachs	7.3	59.2	0.81	7.0	11.8	1.6	78.7	80.7
3	Terrier	9.3	58.1	0.62	10.0	17.2	1.8	75.0	78.2
4	Terrier	10.9	77.9	0.72	9.1	11.7	1.3	76.5	
5	Spitz	11.6	71.7	0.62	10.3	14.5	1.4		
6	Collie	16.6	80.5	0.49	10.5	13.0	1.0	77.6	76.1
7	Collie	20.8	84.4	0.40	10.7	12.7	0.85	74.8	80.1
Average.....		11.8	68.7	0.58	9.1	13.2	1.17	76.8	78.8

TABLE 3

INJECTED 8 CC. PER KGM. WEIGHT	RACE	WEIGHT OF		BRAIN IN PER CENT OF BODY	SPECIFIC WEIGHT	PER CENT WATER IN	
		Body	Brain			Brain	Blood
		kgm.	*grams*				
30 per cent NaCl.....	Spitz	8.3	53.6	0.65	1.043	76.9	78.3
	Spitz	11.3	74.3	0.66		76.8	83.3 (d = 1.051)
50 per cent dextrose.....	Spitz	7.9	54.0	0.68	1.042	76.4	77.8
	Terrier	12.3	73.3	0.60		77.6	80.3 (d = 1.054)
Normal dogs.....	Terrier	5.2	53.8	1.03	1.0378	78.1	
	Terrier	8.1	64.0	0.79	1.0344	77.7	
	Terrier	12.8	79.0	0.62		77.0	
30 per cent NaCl, 5 cc. per kgm.....	Terrier	8.6	73.8	0.86	1.0412	76.1	d = 1.0521
50 per cent glucose, 16 cc. per kgm.....	Terrier	4.4	56.5	1.28	1.0427	77.6	d = 1.0571

line runs parallel to the first one. This will prove that the seemingly wide variation in the brain weight of dogs underlies similar laws as the brain of the albino rat. The value of spitz and poodle brains differ somewhat from the terrier-collie group.

DISCUSSION. It is evident from table 2 that the results which are ob-

tained in the experiments with hypertonic sodium chloride solutions fulfill all the requirements of the old Monro-Kellie-Burrows theory: the diminution in the amount of spinal fluid as demonstrated in Weed's experiments is accompanied by an increase in the amount of blood from 8.3 cc. per 100 grams of brain to 13.2 cc. or in other words the volume of the contents of the skull remains approximately constant, a fact which is also demonstrated by the same relative weight of the brain in the control experiments and in those in which 15 per cent sodium chloride solutions had been injected. The amount of water as determined by the difference in weight of fresh and dried brains is not as considerably diminished as in Ernst's experiments in cats (1930) who found an average of 71.8 per cent of water in the normal controls as compared with 66.7 per cent after injection of 25 per cent NaCl solution. This difference may be explained either by the fact, that Ernst determined the water in sections of the frontal poles and not of the whole brain and, therefore, always had different proportions of gray and white matter with great differences in water (Weil, 1920), or the use of a more concentrated sodium chloride solution together with a longer narcosis, one hour, in the controls which tends to increase the amount of water in the brain (Weil, 1929) may explain the difference.

That the volume of the brain in the 15 per cent sodium chloride experiments is diminished despite the same relative weight as in the 0.85 per cent NaCl controls may be seen from the increase in specific weight (table 3). In vitro experiments, whole hemispheres were kept for three hours at 37° in a mixture of dog's plasma and sodium chloride solution in the same proportion which would theoretically be obtained if 8 cc. of a 15 per cent NaCl solution per kilogram weight would have been mixed with the whole blood of the dog whose brain was used (31 grams brain in 77 cc. plasma plus 10 cc. of a 30 per cent NaCl solution). The specific weight in such experiments was increased from 1.0360 to 1.0435 on the average: the loss in weight was 2.5 per cent. After injections of 30 per cent NaCl in dogs, 5 cc. per kgm. weight, the specific weight of the brains increased from 1.0378 in the controls, which were injected with 0.85 per cent NaCl, to 1.0434 (averages of two experiments).

CONCLUSIONS

1. The amount of blood which is present in the brains of dogs under different experimental conditions was determined by adding collargol to the solution to be injected. After a given time the amount of silver in 1 cc. of blood and in the whole brain was determined, after ashing of both, with the Volhard method. The amount of blood in the brain was calculated from these two silver analyses.

2. The amount of blood which is present in the brain of an average dog of 10 kgm. weight under ether narcosis was determined as 4.8 cc. = 8.3

cc. per 100 grams brain weight. These figures compare with 5.5 grams per 100 grams brain and spinal cord weight combined as found by Ranke, in normal dogs.

3. Following intravenous injections of 15 per cent sodium chloride with collargol the amount of blood was increased to 13.2 cc. per 100 grams brain weight.

4. Following intravenous injections of 30 per cent sodium chloride and 50 per cent glucose solutions without addition of collargol the specific weight of the brain was increased from approximately 1.038 in normal animals to 1.041–43 and the amount of water in the brain had been decreased from an average of 77.6 per cent to 76.9 per cent.

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THE EFFECT OF RESPIRATION ON THE SCRATCH REFLEX

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Received for publication June 12, 1931

Among the multiplicity of factors which contribute to the variability of spinal reflexes, impulses irradiating from the respiratory center have been shown to be of considerable importance (1931). During the course of the investigations bearing on the effects of respiration on the knee jerk, a number of animals were encountered in which the scratch reflex was easily elicited. These were studied intensively and the results revealed clearly that several aspects of the scratch reflex are definitely affected by respiratory activity. The two outstanding facts determined were: first, there is a lowering of the threshold during inspiration; and second, the after-discharge may reappear during inspiration for several respiratory cycles.

In this report we propose to describe experiments in which these phenomena came into prominence, and to discuss their significance in relation to reflex activity.

METHODS AND MATERIALS. Barbitalized dogs and cats, and decerebrated cats were used. The scratch reflex cannot regularly be elicited in animals under the influence of barbital, but occasionally one is met which responds sharply. As a rule the reflex is easily obtained in the decerebrate preparation. It has been our experience that mangy animals under narcosis can be made to scratch more easily than those whose skins are free from parasites.

The scratch movements were registered by use of the string galvanometer, the electrodes being attached to the thigh and hind foot, and also mechanically on smoked paper by a pulley and lever system, the attachment being made at the ankle. Respiration was recorded by a pneumograph and tambour system in some instances, and in others (fig. 2) by a compound level and pulley system.

The animals were placed in the supine position, rigidly fastened down, with the exception of the limb under observation, which was allowed to swing freely.

EXPERIMENTS AND RESULTS. While the phenomena mentioned in the earlier paragraph have been frequently observed and recorded, they

appeared in a very striking fashion in three animals, and it is mainly upon data from them that this paper is based.

The first animal, a dog, was brought into the laboratory for preliminary observation and examination before being given barbital. He was very

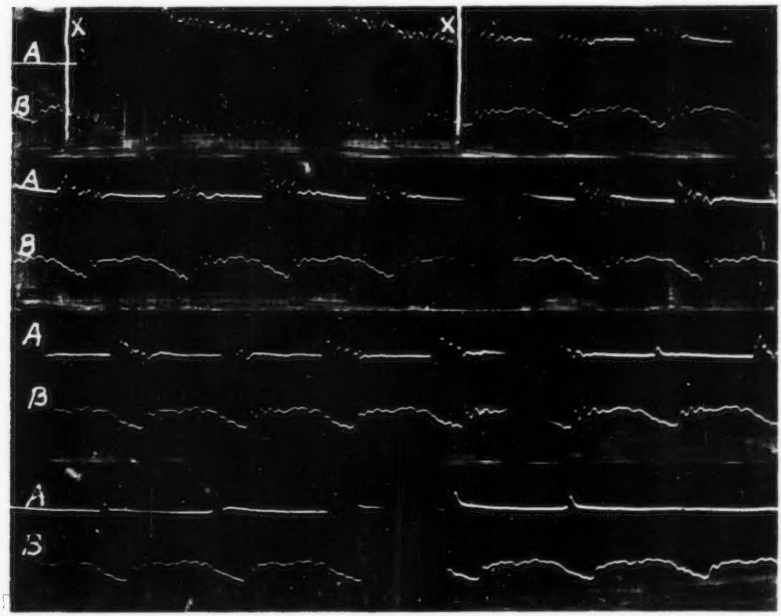

Fig. 1. The inspiratory appearance of the scratch reflex. *A*, record of the scratch movements. *B*, respiration, down stroke inspiration. *xx*, period of stimulation.

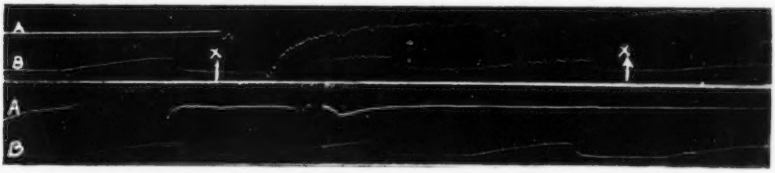

Fig. 2. The inspiratory reappearance of the after-discharge. *A*, record of scratch movements. *B*, respiration, up stroke inspiration. *xx*, period of stimulation.

active, covered with mange, and when not being distracted spent most of his time in scratching, more frequently on the left side. He was then given barbital, and ninety minutes later was again brought into the labora-

tory. He lay quietly, breathed normally, but showed a high degree of reflex excitability. It was noted that the slightest contact with his shoulders, chest, and upper abdomen called forth scratching movements, and that they persisted for a long time. He was then fixed into position for obtaining records. While this preparation was in progress he scratched almost continuously, first on one side and then on the other, but more on the left, just as he had done before narcotization. After fixation into position the scratching activity ceased with the exception of a very occasional weak outburst which subsided rather promptly. The skin was not shaved. The threshold was so low that the mere passing of a camel's hair brush over the receptor field called forth vigorous scratching movements which persisted for some time after cessation of the mechanical stimulus.

Figure 1 is a record from the animal. The stimulus in this instance consisted in rubbing of the left shoulder with the finger. It was applied for almost 12 seconds at a rate of approximately 4 a second. At the onset of the reflex the leg was flexed so sharply at the hip that the string shadow fell outside of the camera slit, but soon relaxed somewhat. During the course of the application of the stimulus the respiratory effects were not definite, but there is some indication of an increase in the intensity of the reaction during the last three inspirations. The stimulus was stopped late in the inspiratory phase. It will be noted that the scratching continued, the first few beats being augmented in intensity, then gradually diminished. With the onset of the next inspiration the beats again increased in intensity. After the lapse of about one minute the foot and leg came to complete rest during the respiratory pause, and finally movements appeared during only a part of the inspiratory phase, and after about one and one-half minutes ceased entirely.

Another animal, a decerebrate cat presented a picture almost identical. This animal had an abrasion on the left cheek, which extended over the neck and left shoulder. It was infested with mange, and the skin injury in all likelihood was the result of repeated scratching. Before decerebration this cat scratched very frequently. After the operation it scratched with each inspiration during the course of the whole afternoon.

Presumably in both of these animals just described a constant stimulus was present, but just below the threshold. In the case of the first animal the augmenting effect of irradiating impulses from the respiratory center was not sufficient to set the mechanism into activity, but after the reflex was once started, was adequate to maintain it for some time, and to lengthen the period of subsidence. In the second animal the constant stimulus was probably nearer the threshold, and the added effect of impulses from the respiratory center was great enough to bring about a response.

The third animal was a dog, not mangy, which after barbitalization

responded sharply to stimulation in the scratch receptor field, but manifested no spontaneous scratching as did the cat just described. Figure 2 is a record taken from this animal. The leads to the galvanometer were so arranged that the downward movement of the string shadow indicates flexion, just the opposite from that obtaining in figure 1. Inspiration is also indicated by the upstroke of the lever instead of the downstroke as in the first illustration.

The stimulus, also mechanical, was applied for approximately 14 seconds at the rate of 4 a second. The onset of the reflex is characterized by an abrupt flexion at the hip and execution of a few vigorous beats at the rate of approximately 5 a second. This was followed by a rapid recession from the flexed position to one of slight relative extension, and the disappearance of the large vigorous beats, to be replaced by much smaller beats and at a more rapid rate. The stimulus was cut off during the respiratory pause. The after-discharge persisted in the form of weak beats at a more rapid rate with the leg remaining in the relatively extended position. During the next inspiration, with a sharp extensor jerk, the leg broke away from the extended position into one of marked flexion and executed a few beats of considerable magnitude, then came to rest. With the next inspiration scratching again appeared at the rate of about 5 a second, but ceased abruptly before the end of the inspiratory act. This was repeated during the next inspiration, but with still shorter duration. With the next inspiration a few feeble beats appeared, followed by a permanent period of quiescence.

The third animal differed from the other two in the absence of a constant stimulus, at least nothing could be found which would account for stimulation. The result portrayed on the record is therefore interpreted as an instance of the inspiratory reappearance of a true after-discharge. We have a number of times observed an augmentation of the after-discharge during inspiration when this phase of respiration fell within the normal after-discharge period, but this is the only instance of a reappearance. To our knowledge no mention is made in the literature of this phenomenon, excepting a brief report by us (1929), in connection with respiration, although Sherrington states that the after-discharge may reappear after a momentary inhibition.

Discussion. It was pointed out in our previous reports dealing with the inspiratory augmentation of reflexes and the activity of skeletal muscles, that the data did not warrant a definite postulation of the mechanism involved. The results with the scratch reflex throw very little if any additional light on this point. It appears quite clear that impulses from the respiratory center, either directly or indirectly, contribute to the excitatory state in the spinal cord, which in turn is manifested by a lowering of the threshold and by an increase in the number of active motor-neurones.

The scratch reflex is a complicated act, the beats under normal condi-

tions being executed on a background of flexion at the hip. The flexion served to bring the limb into a position to make the contact of the foot with the skin effective in removing the source of irritation. In animals which have been narcotized, decerebrated, or made spinal, this flexion, which usually marks the onset of the reflex, is quite variable, and appears to be unessential to the execution of beats. In the after-discharge, and in a gradually dying reflex the beats outlast the flexion, the late augmentatory effect of inspiration manifesting itself merely as reappearing beats.

In the earlier paper (1931) dealing with the inspiratory augmentation of proprioceptive reflexes, the view was taken that the essential process in inspiratory augmentation does not consist in a shortening of the refractory period of some component of the reflex arc. The scratch reflex consists of grouped discharges appearing rhythmically, and on this basis the assumption may be made that the mechanism exhibits a refractory phase, the rate giving an indication of the duration of this state. One would assume that if the effect of irradiating respiratory impulses is that of a shortening of the refractory period, this effect would reflect itself in an increase in the beat rate. Our records reveal variations in rate, but in no case has it been possible to establish a definite coördination with any respiratory phase.

Our studies on irradiating respiratory impulses have not revealed any definitely patterned responses below the levels of the respiratory muscles, nor do they change the patterned responses in which the final paths may be engaged at any time. The effect is merely to augment any activity which happens to be called forth by appropriate stimulation, whether it be the knee jerk, the Achilles jerk, the scratch reflex, shivering, or parathyroid tetany.

SUMMARY AND CONCLUSIONS

1. Experiments have been described in which it was shown that the activity of the respiratory center may be definitely reflected in the scratch reflex.

2. The effects of inspiration on the scratch reflex are manifested in two ways: first, there is an inspiratory lowering of the threshold; and second, the after-discharge is prolonged.

3. On the assumption that the rhythm of the scratch reflex indicates a refractory phase, there is no evidence that this is affected by inspiratory impulses.

4. The pattern of the reflex is not altered, but merely the intensity.

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THE COMPOSITION OF BLOOD IN THE ARTERY, IN THE INTERNAL JUGULAR VEIN AND IN THE FEMORAL VEIN DURING OXYGEN WANT

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Received for publication June 10, 1931

It has been supposed that mountain sickness is related to a diminished oxygen supply to the brain. The evidence for this hypothesis has been indirect insofar as man is concerned. Since the introduction by Myerson and Halloran (1) of a method for obtaining blood from the internal jugular vein, it has become possible to study directly some aspects of metabolic processes in the brain by comparison of the composition of blood from it with that from an artery. Such experiments, with subjects breathing air of normal composition, have been carried out by Myerson and Halloran (2) and by Lennox (3). Under the conditions of their experiments, as much or more oxygen was removed in the cerebral circulation as in the peripheral circulation. It follows that in rest the mean oxygen pressure in cerebral capillaries is no more, probably less, than in capillaries of muscular tissue.

Our experiments have been carried out at the Boston State Hospital on subjects breathing an air-nitrogen mixture containing 9 per cent oxygen, equivalent in respect to oxygen to an altitude of 22,000 feet. While these subjects are mental patients, mainly diagnosed as dementia praecox, there is no reason to suppose from other experiments that their brain processes are pathological in respect to oxygen, carbon dioxide or lactic acid. Each subject was brought to the laboratory 2 or 3 hours after breakfast and placed in a reclining position. After the subject had breathed room air through a face mask for some minutes, a valve was turned to connect him with the gas mixture. If he remained tractable, puncture of the internal jugular vein was begun from 3 to 10 minutes later. As soon as blood began entering the syringe a second man punctured the right femoral vein and a third, the left femoral artery. In most cases blood was entering the three syringes simultaneously; only in 2 or 3 cases was the jugular puncture completed before blood appeared in the other syringes.

Ten such experiments on 10 subjects have been carried out successfully. Two additional experiments failed, one because of improper adjustment of the mask and the other because the specimen of arterial blood was lost.

TABLE 1

Composition of blood taken from femoral artery, femoral vein and internal jugular vein in anoxemia

	H.	C. J.	W. B.	J. P.	J. H.	G. T.	A. F.	J. M.	H. H.	T. O.	AVERAGE
HbO_2 capacity, *vols per cent*	22.25	19.46	17.58	19.40	20.63	19.43	18.63	20.21	20.94	20.00	19.85
O_2 saturation, *vols. per cent:*											
Artery	65	77	48	69	70	63	58	60	63	59	63.2
Jugular vein	46	45	22	51	42	42	42	40	51	35	41.6
Femoral vein	36	56	34	45	42	37	34	34	49	35	40.2
CO_2 content, *vols. per cent:*											
Artery	43.4	39.0	45.3	47.0	43.0	53.2	43.7	45.1	45.4	45.8	45.1
Jugular vein	48.1	48.4	51.4	50.8	51.8	58.3	48.3	48.9	50.7	54.2	51.1
Femoral vein	51.7	52.8	51.5	52.7	53.4	60.1	49.1	52.4	53.5	54.4	53.2
Sugar, *mgm. per 100 cc.:*											
Artery	104	117	113	115	109	95	113	106	107	109	109
Jugular vein	97	95	101	109	94	85	102	101	95	100	98
Femoral vein	100	111	107	109	109	91	109	103	104	106	105
Lactic acid, *mgm. per 100 cc.:*											
Artery	16.8	23.5	23.9	20.3	13.6	29.1	16.0	13.2	12.8	10.8	18.0
Jugular vein	20.3	26.7	21.1	19.2	14.1	26.3	17.2	14.8	12.8	9.6	18.2
Femoral vein	16.0	22.7	20.3	19.2	13.6	27.1	17.9	13.2	14.8	9.6	17.4
Alkaline reserve:*											
Artery		44.6†	43.3	47.3	43.2	49.4	43.6	47.2†	44.8	45.1	45.2
Jugular vein			43.2	46.8	43.1	48.7	44.2		46.1	45.7	45.4
Femoral vein	46.7†	44.3†	43.7	46.7	41.9	48.5	43.6	47.3†	45.2	45.8	45.1
pH_s:											
Artery	7.46	7.50	7.37	7.40	7.39	7.41	7.41	7.50	7.40	7.41	7.42
Jugular vein	7.43	7.39	7.31	7.38	7.28	7.34	7.37	7.40	7.38	7.32	7.36
Femoral vein	7.39	7.30	7.31	7.36	7.24	7.31	7.34	7.41	7.34	7.32	7.33

* Alkaline reserve is defined as the total carbon dioxide content of oxygenated whole blood at a carbon dioxide pressure of 40 mm. Hg.

† Not included in the average.

The blood was protected from *in vitro* changes in the usual way (4). Lactic acid was determined by the method of Friedemann, Cotonio and Schaffer (5) and sugar by the micro method of Folin and Malmros (6). Alkaline reserve was determined by equilibration of whole blood at 37.5° with air containing about 5 per cent carbon dioxide, analysis of the two phases and calculation, by use of the chart of Henderson and associates (7), of the total carbon dioxide content at a partial pressure of carbon dioxide of 40 mm. Hg. Values for pH_s were calculated on the basis of knowledge of the alkaline reserve, carbon dioxide content, oxygen content and the relations between these variables and the hydrogen ion concentration established by Henderson and associates (7) and by Van Slyke and Sendroy (8).

The experimental results given in table 1 indicate that, with a degree of anoxemia at least twice as great as that observed by Barcroft and associates (9) after acclimatization at an altitude of 14,000 feet, metabolic processes in the brain remain aerobic. As much oxygen was removed in the cerebral circulation as in the peripheral circulation. At least $\frac{2}{3}$ as much oxygen was removed in the cerebral circulation in our cases with anoxemia as in cases with normal arterial oxygen content (3). The changes in concentration of lactic acid are equally conclusive. The mean values in blood from the 3 sources are almost identical and in 6 of 10 cases the lactic acid content of arterial blood differed by less than 2 mgm. per 100 cc. from that of blood from the jugular vein. Neither is there any relation between degree of anoxemia and change in lactic acid content in the cerebral or in the peripheral circulation. In fact the subject with the lowest oxygen content in arterial blood (48 per cent of capacity) had less lactic acid in blood returning from the brain than in arterial blood. The most reasonable interpretation of these results is that when the oxygen saturation of arterial blood lies between 48 and 77 per cent there is a normal supply of oxygen to the brain and periphery and no considerable accumulation of lactic acid in either area.[1] Since the oxygen supply is normal it is probable that no other

[1] In 6 of the 10 subjects the concentration of lactic acid was above the usual resting value. This was not due to oxygen lack for there was no relation between arterial saturation and lactic acid concentration. Nor is the duration of anoxemia involved for one of us recently breathed 9 per cent oxygen for 40 minutes with little distress and without previous acclimatization. At the end of that time a sample of arterial blood was 56 per cent saturated with oxygen and contained only 12 mgm. per cent of lactic acid. The work of Tolstoi and associates (10) shows that adrenaline will cause an increase in lactic acid in dogs. This is not a probable explanation of our results for most of the subjects were not much excited if one is to judge by pulse rate and blood sugar concentration. Furthermore we have recently had in the laboratory a boy of 16 in an early stage of dementia praecox. He was obviously quite excited; his resting pulse rate was 170, his blood sugar was 177 and his blood lactic acid was normal, 17 mgm. per cent! When in his usual environment his pulse rate and blood sugar were normal.

fixed acid (such as phosphoric acid from phosphocreatine) is liberated. The observations on alkaline reserve point in the same direction, for its mean value in specimens of blood from the jugular vein differed by only 0.2 vol. per cent from that of arterial blood.

The disappearance of sugar in the cerebral circulation has been noted before (2). In these experiments, with the subjects not in the fasting state, there was a disappearance of sugar in every case, the mean being 11 mgm. per 100 cc. of blood. The mean quantity of oxygen used was 4.3 cc. which would oxidize only 6 mgm. of sugar. It is probable that the remainder was being stored.

From the data given in the table, it is possible to calculate respiratory quotients. All but one of the quotients are above unity due to increased volume of air breathed without a corresponding increase in metabolism. The mean values for R. Q. are 1.36 in the brain and 1.81 in the leg. The difference may be explained by *a*, a greater distribution of capillaries in the leg and hence a more rapid washing out of CO_2, or *b*, a greater buffering by muscular tissue than by nervous tissue so that between given pressures of carbon dioxide less CO_2 will be given off, or *c*, a combination of *a* and *b*. Because of inadequate experimental evidence it is impossible to settle this question now.

The values for pH_s in arterial blood are in some cases more alkaline than normal because of over-ventilation. Blood returning from the brain is more alkaline than blood returning from the tissues in 7 of 10 subjects and has the same reaction in 2 of the other 3. This uniform difference in reaction is related to the fact that with over-ventilation carbon dioxide is swept out from skeletal tissues more rapidly than from the brain. As suggested above, this may be due to a more abundant distribution of capillaries in muscle than in brain or may be due to difference in buffering capacity of the two tissues.

These results are quite at variance with those obtained by Winterstein and Gollwitzer-Meier (11). They found a decrease in alkaline reserve of several volumes per cent in blood during its passage through the cerebral circulation in dogs and rabbits at altitudes of 1560 and 3136 meters. On the basis of this observation they postulated the formation of lactic acid in the brain and its oxidative removal in the pulmonary circulation. The properties of dog's blood are not much different from those of human blood so that it is improbable that the oxygen saturation of dog's blood is below 85 per cent, perhaps not below 90 per cent, at 3136 meters. Accordingly it is difficult to believe that there could have been anaerobic metabolism in the brain under the conditions of their experiments. It is possible that some uncontrolled factor in their experiments was responsible for such surprising results.

Our observations are more in line with those of Gesell and associates (12).

He has come to the conclusion that hydrogen ion concentration can no longer be considered the universal stimulus to respiration but that the metabolic processes in the center itself must be considered. As pointed out frequently by Henderson (13) it is illogical to assign unique functions to a single variable such as $(H)^+$ when variations in it are necessarily associated with changes in concentration of other ions.

Our results fit in very well with those of Cullen and associates (14) who have shown that when patients with heart disease are exercised a large increase in pulmonary ventilation takes place with no increase in acidity of blood passing through the brain. So far as we can judge the increase in pulmonary ventilation which occurs in anoxemia is associated with a normal oxygen supply to the brain and with more alkaline reaction than normal in blood leaving the brain. The stimulus to respiration cannot be defined so precisely as Winterstein and others would suppose.

SUMMARY

Metabolic processes in the brain and in the legs have been studied during partial anoxemia by examination of samples of blood obtained simultaneously from the internal jugular vein, the femoral vein and the femoral artery. The arterial oxygen content ranged from 48 to 77 per cent of capacity and yet there was no accumulation of lactic acid or other fixed acid in either area. Blood leaving the brain was within the normal range in most subjects. The hypothesis of Winterstein and Gollwitzer-Meier that increase in breathing at high altitudes is due to increase in acidity of the respiratory center is not in accord with these facts.

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THE RAPIDITY OF INTERCHANGES BETWEEN THE BLOOD AND LYMPH IN THE DOG

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Received for publication June 12, 1931

Observations on the rapidity with which interchanges take place between the blood and lymph have been confined in the past to thoracic duct lymph. Osato (1921) found that substances of small molecular weight rapidly reached an equal concentration in thoracic duct lymph and blood while those with larger molecules appeared in the lymph in lower concentration. Arnold and Mendel (1927) found that diffusible substances passed easily and rapidly at all times between the blood and the thoracic duct lymph, and that fluctuations occurring in the concentration of constituents of the blood were almost immediately reflected in this lymph. They found a conspicuous difference in the concentration of protein in serum and lymph but observed a constant ratio under a variety of experimental conditions, which, however, did not include measures for the critical reduction of the blood proteins.

Conklin (1930) found that certain foreign proteins, injected into the circulation of the frog, could be detected in the lymph in a remarkably short time. Considering the close anatomical relationship and proximity of the blood and lymph capillaries, she felt that it was extremely doubtful if any great difference could exist between tissue fluid and lymph.

Thoracic duct lymph is obtained largely from the intestines and liver, the capillaries of which are extraordinarily permeable to blood proteins (Starling, 1894). It is a mixture of absorbed fat, highly proteinized lymph from the abdomen, and a small fraction of less proteinized lymph from subcutaneous lymphatics.

Drinker and Field (1931) and Field and Drinker (1931a), working on lymph obtained from subcutaneous lymphatics in dogs and representing reasonably uniform areas of drainage, were forced to the conclusion that tissue fluid and lymph possess an approximate degree of identity. They based this conclusion on the ease and rapidity with which the lymphatics promptly contained all material from the tissue spaces which was either unabsorbable or at best only slightly absorbable by the blood vessels.

Further observations have been made which strengthen this view and show that subcutaneous lymph as well as thoracic duct lymph promptly

reflects alterations in the constituents of blood serum. These observations are of three varieties: *a*, sterile inflammation; *b*, venous obstruction, and *c*, plasmapheresis.

EXPERIMENTAL. Dogs anesthetized with sodium barbital given intravenously or with Nembutal "844" given intraperitoneally were used. The lymphatics of the cervical region, of the lower leg or both were isolated and cannulated, as previously described (Field and Drinker, 1931a), and collections of lymph were made. Protein determinations were made refractometrically.

Sterile inflammation. Whenever the capillaries of the body are injured, whether mechanically or otherwise, they immediately become more per-

TABLE 1

The effect of immersion of the paw in hot water on the amount of protein in lymph flowing from the part

NUMBER OF EXPERIMENT	TOTAL PROTEIN NORMAL LYMPH	PERIOD OF IMMERSION	TEMPERATURE	TOTAL PROTEIN AFTER IMMERSION	
	per cent	*minutes*	*°C.*	*time*	*per cent*
1	0.68	60	100	5 minutes	2.60
				20 minutes	2.89
				1 hour	3.44
2	0.89	2	100	6 minutes	1.13
				20 minutes	2.93
				1 hour	4.00
3	1.59	10	56	5 minutes	1.41
				20 minutes	1.23
				1 hour	1.54
				2 hours	2.37
4	0.58	10	61	4 minutes	1.13
				20 minutes	2.46
				1 hour	4.14

meable to the blood proteins. Okuneff (1924) observed that local heating of the skin which produces peripheral dilatation and rapid flow of blood increased the passage of trypan blue from the blood stream into the heated tissues. Landis (1927) found that there was an increased permeability of the capillary wall of the frog from mechanical injury, and that water of sufficiently high temperature was able to increase the permeability of the capillary wall to the extreme extent of producing stasis. Menkin (1930), in his studies on inflammation, showed that the rapid accumulation of dye in an inflamed area was associated with increased capillary permeability. Underhill and Fisk (1930) were able to show from a study of the distribu-

tion of dyes, that blood concentration following a superficial burn was due to the loss of fluid from the blood through increased capillary permeability, and that the edema fluid produced under such conditions so closely resembled blood serum as to be regarded as blood plasma.

In the first series of experiments on inflammatory lymph, the large lower leg lymphatic was cannulated at the ankle. After a series of protein determinations had been made on the normal leg lymph, the entire paw was immersed in water above 56°C. for varying lengths of time. During and after the immersion protein determinations were made.

Table 1 gives four typical experiments of this series, in which it can be seen that the capillaries are rendered abnormally permeable to the blood proteins almost at once. The lymph immediately reflects this change in permeability by an increased protein content, which continues for some

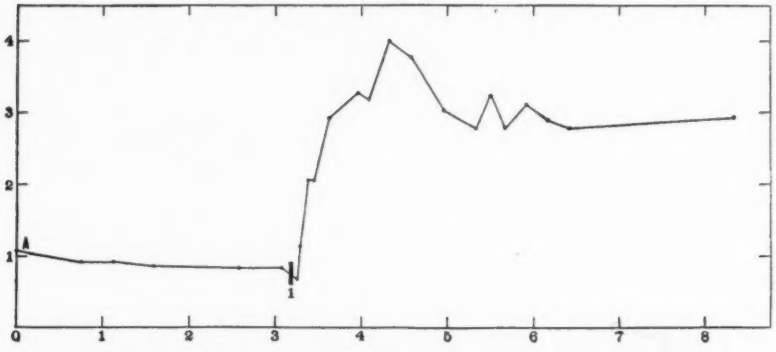

Fig. 1. The effect of a sterile inflammation on the protein content of subcutaneous lymph. *Ordinates*, per cent of protein; *abscissae*, time in hours. *A*, leg lymph. At mark *1*, the paw was immersed in water at 100°C. for 2 minutes.

time since the capillaries are probably permanently damaged. The flow of lymph from the leg is tremendously increased under such conditions. In one experiment 15.0 cc. were collected in $3\frac{1}{2}$ hours following the immersion, while ordinarily it is not possible to collect more than 0.5 to 1.0 cc. during such a period.

Figure 1 shows a typical experiment of long duration in which the lymph very promptly reflects the capillary damage.

Normally there are few if any cells in lymph taken from the lower leg lymphatics (Haynes and Field, 1931), but in sterile inflammatory lymph it is usually possible to find a few lymphocytes and polymorphonuclear leucocytes.

Venous obstruction. Emminghaus (1873) observed that lymph obtained from leg lymphatics became bloody shortly after ligation of the veins of

the leg. He thought that bloodiness of lymph should be associated with an increased protein concentration since, presumably, any change in the capillary or lymphatic wall sufficient to permit the passage of erythrocytes would allow protein molecules to pass through with even greater ease. Contrary to his expectation he found that in practically every case the increased lymph resulting from venous obstruction had a much lower protein content. Starling (1894) found that when the portal vein was obstructed, increasing the pressure in the intestinal capillaries, there was, in every case, a great increase in lymph flow from the thoracic duct but that at the same time the lymph was more watery. It is a well-known fact that the edema fluids of blood stasis obtained under clinical conditions are usually low in protein. Simple filtration edemas are frequently observed in normal subjects. Thompson, Thompson and Dailey (1928) observed in individuals who stood still for 20 to 30 minutes increases in red cell count and total red cell volume, indicating concentration of the blood and corresponding increases in the protein content of the plasma. They believed the fluid lost was approximately protein free. Harrop and Waterfield (1930) observed a 14 per cent diminution in blood volume in the erect posture, but found that 60 per cent of the total blood protein left the circulation in company with the diffusible constituents.

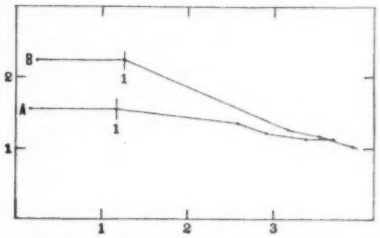

Fig. 2. The effect of venous obstruction on the protein content of leg lymph. *Ordinates*, per cent of protein; *abscissae*, time in hours. *A*, left leg lymph; *B*, right leg lymph. At mark *1*, all veins at ankle and in groin were occluded.

We have found no measurements of the actual changes in the protein concentration of subcutaneous lymph under conditions of venous obstruction and the rapidity with which these changes occur except in the paper by Emminghaus (1873).

Figure 2 illustrates a typical experiment of this series, the protocol of which is as follows:

Experiment 40. April 13, 1931. Dog. Weight 20.7 kgm.

10:30 a.m. 0.8 gram Nembutal "844" given intraperitoneally.
11:30 a.m. Main lymphatics of right and left legs cannulated at the ankle. All veins at ankle and groin exposed.

	Left leg lymph	*Right leg lymph*
12:10 p.m.	1.55 per cent protein Occasional red cell present	
12:15 p.m.		2.25 per cent protein
1:10 p.m.	1.55 per cent protein All veins ligatured	

1:15 p.m.		2.25 per cent protein All veins ligatured
2:30 p.m.	Lymph slightly red 18 to 35 r.b.c. to a field (high dry), occasional small and large lymphocytes and leucocytes	
2:35 p.m.	1.35 per cent protein	
2:55 p.m.	1.21 per cent protein	
3:07 p.m.	Femoral artery tied	
3:10 p.m.		1.26 per cent protein
3:22 p.m.	1.14 per cent protein Red cell content similar to 2:30 specimen	
3:32		1.16 per cent protein
3:55		1.02 per cent protein

This experiment confirms Emminghaus and shows that diapedesis of red cells during venous obstruction is not associated with an increased protein content of lymph. Other experiments in this group were carried out on cervical lymph. All of the veins at the base of the neck were carefully exposed and tied or clamped. The resulting increased cervical lymph invariably contained less protein.

Plasmapheresis. The rapidity with which lymph protein concentrations follow blood protein concentrations is well illustrated by plasmapheresis.

The dogs were prepared by ligation of all lymphatic entrances into the veins. The right and left cervical lymphatics and the thoracic duct were cannulated. Blood samples were taken from a femoral artery and reinjections of washed dog red cells suspended in Locke's solution or physiological saline were made through a femoral vein. Arterial blood pressure was recorded throughout the experiments. Figures 3 and 4 illustrate typical experiments.

The first effect of reducing blood protein noticed in plasmapheresis experiments is a marked increase in lymph flow from all cannulated vessels. This may be surprisingly great. For example, in the experiment from which figure 4 was constructed 800 cc. of lymph flowed from the thoracic duct in 4½ hours. This lymph invariably becomes somewhat bloody during the period of observation, but the subcutaneous lymph remains clear and watery and is surprisingly plentiful.

Figures 3 and 4 show the changes in blood and lymph proteins which occurred in two characteristic experiments. In the first instance, figure 3, 2160 cc. of blood were removed and 3000 cc. of red cells suspended in saline replaced the loss. The systolic blood pressure in the animal was 140 mm. of mercury at the start of the experiment and at the close, 4 hours and 25 minutes later, was 110 mm. of mercury. The protein concentration of the thoracic duct lymph follows the fall and rise in blood protein with a fair degree of evenness. In table 2 are found the ratios of

blood protein to lymph protein throughout the experiment. There is a tendency toward the maintenance of the original ratio which is broken for brief periods by the hemorrhage and infusion of red cells in salt solution.

Figure 4 presents a more radical experiment. In this case 2550 cc. of blood were removed and 3950 cc. of red cells in salt solution were injected. The systolic blood pressure at the beginning of the experiment was 150

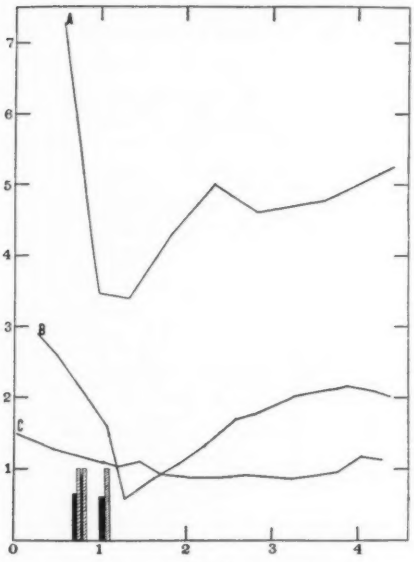

Fig. 3

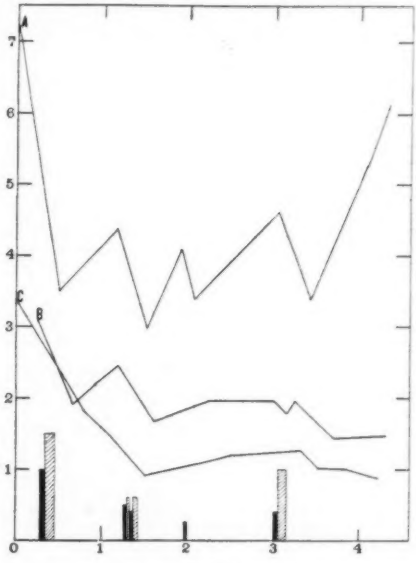

Fig. 4

Fig. 3. The effect of plasmapheresis on the protein content of blood, thoracic duct lymph, and cervical lymph. *Ordinates*, per cent of protein; *abscissae*, time in hours. *A*, blood serum; *B*, thoracic duct lymph; *C*, cervical lymph. Solid black portions represent amount of blood withdrawn; cross-hatched portions represent volume of cells suspended in salt solution re-injected. (From 0 to 1 on ordinates = 1000 cc.)

Fig. 4. The effect of plasmapheresis on the protein content of blood, thoracic duct lymph, and cervical lymph. *Ordinates*, per cent of protein; *abscissae*, time in hours. *A*, blood serum; *B*, thoracic duct lymph; *C*, cervical lymph. Solid black portions represent amount of blood withdrawn; cross-hatched portions represent volume of cells suspended in salt solution re-injected. (From 0 to 1 on ordinates = 1000 cc.)

mm. of mercury and at the close was 110 mm. of mercury. The rapid increase in blood protein at the close of the experiment is probably due to loss from the vessels of most of the salt solution introduced in the final infusion, rather than to diffusion of protein into the capillaries from the tissues. In table 3 are found the ratios of blood protein to lymph protein

in this experiment. Again there is a tendency for ratios to hold in the neighborhood of the original value even though the withdrawals of blood and infusions of washed corpuscles were exceedingly large.

Morawitz (1906) and Hirota (1928) suggested that, following severe hemorrhage, protein from the tissue fluid may enter the blood capillaries directly instead of via the lymphatic route. Our experiments do not answer this question. Field and Drinker (1931b) showed that when horse serum is injected subcutaneously in dogs in which the blood protein has

TABLE 2

Ratio of blood protein to thoracic duct and cervical lymph protein in experiment 1, figure 3

TIME	PER CENT BLOOD PROTEIN / PER CENT THORACIC DUCT LYMPH PROTEIN =	PER CENT BLOOD PROTEIN / PER CENT CERVICAL LYMPH PROTEIN =
minutes		
0	7.3 / 2.8 = 2.60	7.3 / 1.4 = 5.21
25	3.4 / 1.6 = 2.11	3.4 / 1.1 = 3.09
45	3.4 / 0.6 = 5.65	3.4 / 1.0 = 3.4
75	4.3 / 1.1 = 3.90	4.3 / 0.94 = 4.57
105	5.0 / 1.7 = 2.94	5.0 / 0.89 = 5.62
135	4.6 / 1.8 = 2.53	4.6 / 0.91 = 5.1
180	4.7 / 2.01 = 2.33	4.7 / 0.86 = 5.47

TABLE 3

Ratio of blood protein to thoracic duct and cervical lymph protein in experiment 2, figure 4

TIME	PER CENT BLOOD PROTEIN / PER CENT THORACIC DUCT LYMPH PROTEIN =	PER CENT BLOOD PROTEIN / PER CENT CERVICAL LYMPH PROTEIN =
minutes		
0	7.3 / 3.36 = 2.17	7.3 / 3.08 = 2.37
30	3.5 / 2.4 = 1.46	3.5 / 2.4 = 1.46
65	4.38 / 2.42 = 1.79	4.38 / 1.37 = 3.19
87	2.97 / 1.85 = 1.6	2.97 / 0.92 = 3.22
115	4.1 / 1.8 = 2.28	4.1 / 1.02 = 4.1
125	3.39 / 1.87 = 1.81	3.39 / 1.07 = 3.39
183	4.62 / 1.9 = 2.42	4.62 / 1.25 = 3.69
206	3.39 / 1.75 = 1.93	3.39 / 1.15 = 2.94
245	5.25 / 1.45 = 3.61	5.25 / 0.92 = 5.7

been greatly lowered by plasmapheresis, horse protein does diffuse directly into the blood capillaries. In that case the extravascular protein concentration was much higher than the concentration of the blood protein. It would be of interest, in dogs with all lymphatic entrances into the blood stream ligated, to bleed radically and inject horse serum subcutaneously which had been diluted with salt solution so as to have a protein concentration equal to that of the lymph in the injected region. If, under these circumstances, horse protein was found in the blood stream it would indicate that the assumption of Morawitz and Hirota was correct.

DISCUSSION. Changes in the protein content of the blood, whether due to an injured capillary wall or to a great reduction in the circulating blood proteins, are almost immediately reflected in an alteration in the protein concentration of the lymph. These changes can only mean that the capillaries of the dog are partially permeable to protein molecules and that, along with the passage of the diffusible constituents, there is a passage of the less diffusible colloids.

When the capillaries of an area are injured, increased permeability invariably results and as a consequence the lymph draining this area contains almost as much protein as the plasma itself. The passage of this protein through the capillary endothelium, through the tissue space and into the lymph occupies such a short time between the onset of inflammation and its collection from a large drainage lymphatic that it becomes increasingly difficult to see how, under such circumstances, the actual transudate from the blood could be greatly modified during its passage through the tissue spaces. The rapidity of the changes during plasmapheresis is equally striking.

Under conditions of venous obstruction, the subcutaneous lymph again reflects the conditions which must exist in the tissue spaces. An increase in capillary pressure induces filtration and hinders absorption of water. Krogh (1929) has pointed out that the filtration of colloids is undoubtedly slower than that of crystalloids. A tissue fluid low in protein is reflected in a dilute lymph.

SUMMARY

1. Following a sterile inflammation, the capillaries of the dog become abnormally permeable to the blood proteins, with the result that the protein content of subcutaneous lymph increases markedly at once.

2. Venous obstruction causes an outpouring of very dilute subcutaneous lymph in which erythrocytes are usually found.

3. Plasmapheresis is accompanied by a marked depletion in the protein content of thoracic duct and subcutaneous lymph. The flow of lymph is very great after plasmapheresis, and the lymph collected must flow from blood capillaries, through the tissues, and into the lymphatics with very little change in composition.

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THE PRODUCTION OF PLACENTOMATA IN YOUNG RATS FOLLOWING GONADAL STIMULATION WITH PITUITARY IMPLANTS[1]

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Received for publication June 16, 1931

In an analysis of the factors involved in puberty and accelerated genital development following endocrine treatment, (Engle, 1931a, b) it was thought advisable to test the physiological development of the uterus of immature rats by means of induced placentomata. Artificial production of placentomata in the rat by Frank (1911) whose work was corroborated later by Corner and Warren (1919) extended the observations of Loeb (1908) on the guinea pig, to this animal. Long and Evans (1922) introduced a modification of Loeb's original technic of cutting the uterus, by threading the uterus so that the thread passed through the uterine lumen, and then tying a loose loop, thereby avoiding profuse bleeding and marking the exact point of injury. Their efforts to evoke a deciduous growth by threading uteri of rats during any phase of normal 4–5 day oestrous cycle were unsuccessful. However, when they stimulated the uteri of pseudopregnant, or of lactating rats, their results were uniformly positive. The fact that corpora lutea persist longer during the condition of pseudopregnancy and of lactation than during normal 4–5 day cycles, lead Long and Evans to conclude that stimulation of artificial placentomata necessitated the presence of corpora lutea of an age corresponding to the normal time interval between ovulation and subsequent implantation, in the rat about four days.

Teel (1926) demonstrated the production of placentomata in the uteri of normal 4–5 day cycle rats, after the prologation of the life of the corpora lutea by daily injections of a sodium hydroxide extract of beef pituitary. Weichert (1928) provoked placentomata in castrated rats and guinea pigs whose uteri were sensitized to lutein extracts, purified fractions of which were reported in 1930 by Hisaw, Fevold and Meyer, by preparatory injections of oestrin. From these and various other investigations, the production of artificial placentomata has been accepted as a manifestation of proper physiological progestational conditioning of the uterine mucosa.

[1] Aided by a grant from the National Research Council Committee for Research in Problems of Sex, administered by Dr. P. E. Smith.

In a recent series of investigations by Engle (1931a, b) on the relationship of first oestrus, mating and ovulation in young female mice after anterior lobe implants, it was noted in the treated animals that 1, vaginal orifices were established between the third and fourth day of treatment; 2, the first oestrus occurred one to two days later; 3, that mating responses at first oestrus were found in more than half of the animals; 4, that not all these ovulated, and 5, that fertilized ova were not found implanted in the uterine mucosa.

The biological criterion of true sexual maturity, natural or precocious, is obviously the capacity for complete reproductive activity (Engle, 1931). In view of the fact that the ova of the pituitary treated animals were fertilized, although no implantations were observed, and that the ovary appeared normal, it was suggestive that although the uterus appeared normal, microscopically, it might not be physiologically mature. The object of these investigations was to test the physiological state of the uterus of precocious sexually developed female rats.

EXPERIMENTAL PROCEDURE. The method employed in testing the physiological maturity of the uteri of precocious sexually developed rats consisted of induction of a precocious sexual state in immature rats, subsequent prologation of the life of corpora lutea, by daily injections of basic (NaOH) extract of fresh beef pituitary and stimulation of the uterus after such preliminary treatment. Controls consisted of immature females which were treated identically, except that daily injections of follicular hormone (amniotin-Squibb's)[2] were substituted for the anterior pituitary implants.

A group of thirty-three normal 18 and 19 day female rats, ranging from 30 to 46 grams in weight (calculated average 38.+ grams) were used (table 2). Twenty animals were used experimentally, and the remaining thirteen were controls. The rats were from the laboratory colony stock and of vigorous health. They were weaned at the regular age—22 days—and after weaning were maintained on the normal colony diet which has an adequacy of vitamins. In this way the question of spontaneous placentomata from vitamin deficiency (Bishop and Morgan, 1928) was controlled. The experimental animals received daily intramuscular implants of a single fresh rat pituitary, after the technic of Smith (1926) for 3 to 4 days. A small group of pituitary donors were castrated; the majority, however, were normal mature rats. The treatment was followed by daily intraperitoneal injections of sodium hydroxide extracts of fresh beef pituitary (Teel, 1926). Stock solution of the extract was made every three or four days, in order to avoid contamination or loss of potency. A very small group of animals received injections of 1 cc. of extract per day, which roughly

[2] The amniotin was furnished through the courtesy of Dr. J. F. Anderson, Scientific Director of the E. R. Squibb & Sons Biological Laboratories.

corresponds to one beef gland; the greater share received half as large a dosage, which was ample enough to produce large corpora lutea. On the fourth or fifth day after the injections of basic extract of anterior pituitary were started, the uteri of the animals were stimulated after the method of Long and Evans—namely, threading the uterus. In order to lower the probabilities of failing to injure the uterine mucosa, because of the small diameter of the horns, three to six positions were stimulated in one horn; the second was uninjured. Injections of the basic extract were continued and the animals sacrificed on the third to fifth day after the operation. The genital tracts were inspected macroscopically, and then fixed in Bouin's picro-formal-acetic fixative.

The control series was injected with oestrogenic hormone (Squibb's Amniotin) in place of the original fresh anterior pituitary implants. Subsequent treatment was identical to that of the experimental animals. The control rats received daily injections of 3 rat units (as standardized by Squibb) of amniotin for a period of two to four days. Following this period of treatment, the rats were injected with basic extract of fresh beef anterior lobe from the same stock solutions as was used for experimental animals. The dosage was the same as that of the experimental animals. Uteri of the rats were threaded on the fourth or fifth day of basic extract treatment. The animals were autopsied about four days after the operation. Macroscopic examination of the genital tracts revealed no deciduous growths. The ovaries and the uteri in the regions of stimulation were fixed for microscopic study. (Table 1 shows the treatment of individual animals.)

The results showed the capability of uteri of precocious sexually developed rats to develop deciduomata after stimulation. The microscopic examinations of the genital systems were uniformly positive. The ovaries of all the animals contained corpora lutea, and the greater portion were of the "mulberry" type, as described by Long and Evans (1922). The uteri showed definite deciduous enlargements, varying in size from slight thickening to giant bulbous growths. A few of the genital tracts were studied microscopically, and the gross observations checked. It was of interest, that although the growth of placentomata was uniformly positive, the vaginal orifices of all the animals were not established at the time of autopsy. (See tables 2 and 3.)

The controls were uniformly negative as far as macroscopic examination revealed. The ovaries contained corpora lutea, but the uteri revealed no enlarged decidual areas. Microscopic examinations were made of all control animals and were in general corroboratory. Two cases were found in which the threading was unsuccessful and the uterine mucosa was not injured; these animals are not incorporated in the results. Two cases were recorded (W5813, BH6055) which exhibited small areas of decidual

TABLE 1

Daily treatment of experimental and control animals

NUMBER OF ANIMAL	DAY OF TREATMENT												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Experimental series													
W 5810	ic	ic	ic	ic	x_1	x_1	x_1	x_1S	x_1	x_1	x_1	x_1	A
W 5811	ic	ic	ic	ic	x_1	x_1	x_1	x_1S	x_1	x_1	x_1	x_1	A
W 5812	ic	ic	ic	ic	x_1	x_1	x_1	x_1S	x_1	x_1	x_1	x_1	A
W 6006	i	i	i	—	x	x	x	x	xS	x	x	x	A
W 6007	i	i	i	—	x	x	x	x	xS	x	x	x	A
W 6010	i	i	i	—	x	x	x	x	xS	x	x	x	A
W 6011	i	i	i	—	x	x	x	x	xS	x	x	died	
B 6012	i	i	i	—	x	x	x	x	xS	x	x	x	A
B 6013	i	i	i	—	x	x	x	x	xS	x	x	x	A
W 6014	i	i	i	—	x	x	x	x	xS	x	x	x	A
GH6015	2i	i	i	x	x	x	x	xS	x	x	x	A	
G 6016	2i	i	i	x	x	x	x	xS	x	x	x	A	
W 6026	i	i	i	i	x	x	x	xS	x	x	x	x	A
B 6028	i	i	i	i	x	x	x	xS	x	x	x	x	A
BH6039	i	i	i	—	x	x	x	xS	x	x	x	x	A
B 6040	i	i	i	—	x	x	x	xS	x	x	x	x	A
W 6045	i	i	i	—	x	x	x	xS	x	x	A		
BH6046	i	i	i	—	x	x	x	xS	x	x	A		
BH6047	i	i	i	—	x	x	x	xS	x	x	A		
Control series													
W 5813	3RU	3RU	x	x	x	x	xS	x	x	x	x	A	
G 5814	3RU	3RU	x	x	x	x	xS	x	x	x	x	A	
W 5815	3RU	3RU	x	x	x	x	xS	x	x	x	x	A	
W 5849	3RU	3RU	3RU	3RU	x	x	x	x	x	x	x	x	A
W 5850	3RU	3RU	3RU	3RU	x	x	x	xS	x	x	x	x	A
W 5851	3RU	3RU	3RU	3RU	x	x	x	xS	x	x	x	x	A
W 5852	3RU	3RU	3RU	3RU	x	x	x	xS	x	x	x	x	A
W 5853	3RU	3RU	3RU	3RU	x	x	x	xS	x	x	x	x	A
W 5854	3RU	3RU	3RU	3RU	x	x	x	xS	x	x	x	x	A
B 6053	3RU	3RU	3RU	—	x	x	x	xS	x	x	x	A	
B 6054	3RU	3RU	3RU	—	x	x	x	xS	x	x	x	A	
BH6055	3RU	3RU	3RU	—	x	x	x	xS	x	x	x	A	
B 6056	3RU	3RU	3RU	—	x	x	x	xS	x	x	x	A	

Log: ic = single intra-muscular implant of fresh anterior pituitary of castrated rat.
i = single intra-muscular implant of fresh anterior pituitary of rat.
2i = double implant of fresh anterior pituitary of rat.
x_1 = 1 cc. injection (intraperitoneal) of basic extract of beef anterior pituitary, where 1 cc. = 1 beef gland.
x = 0.5 cc. injection (intraperitoneal) of basic extract of beef anterior pituitary, where 1 cc. = 1 beef gland.
3RU = injection of 3 rat units of amniotin in distilled water.
S = stimulation of uterus.
— = no treatment.
A = autopsy.

TABLE 2

Tabulation of the experimental and autopsy data

NUMBER OF ANIMAL	EXPERIMENT BEGUN					AUTOPSY RECORD		
	Weight	Age	Weaning weight	Stimulation age	Vaginal orifice	Weight	Age	Placentomata diagnosis
Experimental								
	grams	*days*	*grams*	*days*		*grams*	*days*	
W 5810	38	18		25	+	78	31	++*
W 5811	37	18		25	+	81	31	++
W 5812	37	18		25	−	86	31	++
W 6006		19	45	27	+	82	32	+
W 6007		19	48	27	−	70	32	+
W 6010		19	44	27	−	74	32	+
W 6011		19	44	27	−		31	+
B 6012		19	40	27	+		32	+
B 6013		19	33	27	+	74	32	+
W 6014		19	40	27	−	69	32	+
GH6015		19	60	26	+	82	31	+
G 6016		19	56	26	+	79	31	++
W 6026		18	51	25	−	62	30	+
B 6028		18	44	25	−	62	30	+
BH6039		18	60	25	−	80	30	+
B 6040		18	56	25	+	78	30	+
W 6045		18	48	25	−		28	++
BH6046		18	46	25	−		28	+
BH6047		18	45	25	−		28	+
Controls								
W 5813	46	18	58	24	+		29	±
G 5814	41	18	54	24	+		29	−
W 5815	42	18	54	24	+		29	−
W 5849	30	18		25	+	68	30	−
W 5850	32	18		25	+	76	30	n.b.
W 5851	30	18		25	+	70	30	−
W 5852	34.5	18		25	+	69	30	−
W 5853	30.5	18		25	+	70	30	−
W 5854	30	18		25	+	72	30	n.b.
B 6053		18	47	25	+	50	29	−
B 6054		18	46	25	+	54	29	−
BH6055		18	50	25	+	60	29	±
B 6056		18	48	25	+	62	29	−

* ++ designate: very large macroscopic deciduomata.
+ designates: macroscopic deciduomata.
± designates: slight microscopic decidual areas.
− designates: no microscopic decidual areas.
n.b. Show no uterine injury.

cells in regions of the uterine stimulation. The growth of these areas, however, was not extensive enough to distort the circular cross-section of uterus.

DISCUSSION. If the capability to produce placentomata is established as a criterion of physiological sexual maturity of the uterus, the results demonstrate that the uteri of precocious sexually developed rats possess certain criteria of physiological maturity. The controls which received the proper "conditioning" for placentomata reaction in adults—namely, sensitization by follicular hormone followed by luteinization (Weichert, 1928), were generally negative. However, although the capability of precociously developed rats to produce placentomata has been demonstrated, the mechanism has not been explained. There are two angles of question in exploring the mechanism. First, it is of importance to find out whether

TABLE 3

Summary of autopsy data

	EXPERIMENTAL ANIMALS		CONTROLS	
	Positive	Negative	Positive	Negative
Vaginal orifices	8	11	13	0
Macroscopic:				
Ovary: corpora lutea	19	0	13	0
Uterus: placentomata	19	0	0	13
Microscopic:				
Ovary: corpora lutea	4*	0		
Uterus: placentomata	4*	0	(±)2	11†

* Only 4 positives were studied microscopically to check to gross findings.

† W5850 and W5854 of controls showed no uterine injury and are disregarded.

the failure of the control series to develop placentomata was due to lack of induction of precocious genital changes, or to other causes. Second, is the production of placentomata a manifestation solely of proper uterine conditioning (oestrin followed by lutein) or of maturity plus such conditioning.

The effect of oestrin on the infantile uterus has been amply demonstrated (Allen, 1923, etc.). The general failure of oestrin to act on the ovaries has been reported by various workers (Allen et al., 1924). Teel (1926) has demonstrated the action of basic extract of anterior lobe on the uterus through the stimulation of lutein development of the ovary. The question relative to the failure of the control animals to produce placentomata resolved itself into proving whether or not the injections of basic extract of anterior pituitary stimulated proper luteinization, and properly pre-

pared the uterus for progestational proliferation. Histological studies of the ovary reveal large corpora lutea, nevertheless, the possibility that although the corpora lutea are present, they are developed in immature ovaries, or ovaries stunted by previous injections of oestrin (Leonard, Hisaw, Meyer, 1931), and consequently may not be secreting any or enough lutein (progestin) to condition the uterus.

The work of Weichert (1928) who produced placentomata in adult castrates, after proper uterine sensitization, has been noted. The condition of the castrates, whether they were long time castrated with greatly degenerated genital tracts or not, was not reported. Nevertheless, the castrates were adults and had matured normally before castration, and treatment administered by Weichert merely *reëstablished* a proper condition. The condition in the infantile uterus is somewhat different. Further experiments are in progress to test the quality of uterine response in immature animals.

The author expresses his thanks to Dr. E. T. Engle for his suggesting the problem, and also for his invaluable advice.

SUMMARY

1. The production of placentomata in young rats following gonadal stimulation by pituitary implants and subsequent injections of basic extract of anterior lobe was uniformly successful.

2. Controls which received amniotin (Squibb's) in place of fresh anterior pituitary implants but identical other treatment were negative.

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VASCULAR TONE AND VARIATIONS IN LEUCOCYTE COUNT IN THE SYMPATHECTOMIZED DOG

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Received for publication June 19, 1931

Our previous studies on man (1), (2), have indicated that the fluctuations in the leucocyte counts reported by several investigators (3), (4), (5), are due to vasomotor variability by which leucocytes, especially of the polymorphonuclear variety, are alternately liberated from and trapped in the capillaries. In a state of complete relaxation, resting in the recumbent position, the leucocyte count falls rapidly and progressively until after a sufficient time, usually less than one hour, the count will reach a minimum which for most human individuals lies between 5000 and 6000 leucocytes per cubic millimeter of blood. This level we have designated the "basal level." For most individuals pursuing the ordinary routine work of a medical school laboratory the "activity level" of the leucocyte count averages around 9000, while severe physical effort may raise the count to very high levels—20,000 to 35,000 having been noted in athletic competition.

Dogs show similar variations, activity levels around 11,000 to 14,000 are the rule; when trained to rest the count falls in half an hour to an average of 7000; subsequent exercise, especially if coupled with excitement, raises the count suddenly and induces a definite leucocytosis, in which the count exceeds the ordinary "activity level." The leucocyte count may thus be used as an index of the vascular lability since it varies with vasomotor changes.

We are indebted to Prof. W. B. Cannon and to Dr. S. L. Clark for the courtesy and coöperation which placed at our disposal one of the dogs operated upon by Professor Cannon, who had removed the thoracic and abdominal sympathetic chains to the level of the inferior mesenteric ganglion in 1929. It was anticipated that in this animal vasomotor variability would certainly be less than in the normal dog and that correspondingly the fluctuations in the leucocyte count would be less than in normal dogs subjected to procedures designed to elicit variations. This proved to be the case.

At the outset we were faced with a technical difficulty of significance. Ordinarily blood for counts has been obtained from a slight cut in the ear,

or a puncture of the upper lip. By such procedures on the sympathectomized dog we failed to get a sufficiently large drop of blood to fill the hemocytometer pipette, and when a larger incision was made the blood clotted before a sufficient quantity oozed from the wound. We were therefore forced to resort to venipuncture to obtain blood samples. The counts obtained in this way have been found, in a large series of experiments on normal animals, to be identical with those on blood obtained from the ear, lip or foot-pad. Venepuncture, therefore, was adopted as the routine method of obtaining samples.

This observation is interpreted as an indication that subsequent to and in spite of the removal of the sympathetic nerve chain the tone of the peripheral vessels is maintained; in fact, is greater than that of the normal

TABLE 1

Leucocyte counts on the sympathectomized dog, showing the effects of ordinary activity, rest and exercise. Total number of leucocytes per cubic millimeter of blood obtained by venipuncture

	NORMAL ACTIVITY LEVEL	AFTER HALF AN HOUR REST BASAL LEVEL	AFTER EXERCISE
1	10,480	8,080	10,400
2	10,600	8,000	10,800
3	12,200	8,600	12,400
4	10,800	8,680	10,980
5	11,000	8,500	11,120
6	10,680	8,200	10,600
7	10,800	8,060	11,000
8	11,200	8,000	11,000
9	10,500	7,800	10,260
10	10,680	8,080	10,800
Average.....	10,914	8,200	10,952

animal which bleeds freely from an ear incision or a lip puncture. The ears of the dog were pale and cool; furthermore they did not redden or become warm with exercise sufficient to produce severe panting as Schiff (6) long ago showed was the case in normal dogs. When to the respired air carbon dioxide, to the extent of 5 per cent and 10 per cent was added, there was no flushing of the ears, again demonstrating a surprising lack of response on the part of the peripheral vessels.

Before testing the effects of exercise the dog was trained to lie quietly for long periods of time and to submit to venepuncture without the slightest struggle. Leucocyte counts were made when the animal was brought into the laboratory from the kennel and designated "normal activity level," after one-half hour of absolute rest, the "basal level," and after play and exercise severe enough to cause vigorous panting. The results are given

in table 1. In view of the distribution of the counts over several weeks the constancy of the figures is striking. If we compare these counts we find that the normal activity level is in general considerably higher than in this sympathectomized animal, varying in different animals from 11,000 to 15,000 and fluctuating widely in a given normal animal in a similar way. The basal or more accurately the resting level of the leucocyte counts is higher than in normal trained animals which show an average resting leucocyte count of approximately 7000. Thus normally in the dog rest decreases the leucocytes by 50 per cent, while after sympathectomy the reduction is only around 20 per cent. This fact alone would suffice to establish the fact that after sympathectomy there is a marked decrease in vasomotor reactions and a relative stability of the minute peripheral blood vessels.

This is even more strikingly brought out by the counts after exercise. The severest exercise to which the sympathectomized dog would submit after rest raised the leucocyte count to the normal activity level, but not above it in a single instance. This rise of 25 per cent is in sharp contrast with the reaction of normal dogs which, starting from a lower resting level of 7000, may have a rise of 100 per cent or more, establishing a definite physiological leucocytosis of exercise.

Nine series of differential leucocyte counts were made on the sympathectomized animal. These will not be tabulated in detail but the averages show that in the resting state the polymorphonuclear percentage was 67 and the lymphocyte percentage 29. Exercise following rest brought the ratio of polymorphonuclears exactly to the activity level of 72 per cent. Since in previous work we have established the fact that mild exercise increases the ratio of polymorphonuclear leucocytes in normal individuals while severe exercise may give a preponderance of lymphocytes, the slightly altered ratios in the sympathectomized dog again show that the mechanisms for the liberation of leucocytes, whether vascular or splenic, are absent after sympathectomy.

Rebreathing or administration of carbon dioxide to normal resting individuals and to animals always raised the leucocyte count to a figure definitely above the activity level. In the case of the sympathectomized dog a half hour of rest on a hot day, the animal panting vigorously, brought the count down 10 per cent to 9000; four minutes' administration of 5 per cent carbon dioxide raised the count to 10,000 again. In two subsequent experiments the animal breathed air with 10 per cent carbon dioxide. The counts were as follows: In one experiment the activity level was 12,400, after a half hour of rest 8800, after two minutes breathing the carbon dioxide, 12,000. In the second experiment prior to rest 12,200, after rest 9000, after two minutes of 10 per cent carbon dioxide, 12,120. Breathing carbon dioxide even to definite distress of the animal did not raise the

leucocyte count above the activity level. Although a definite effect was produced, the combination of the carbon dioxide and the nervous disturbances did not produce the marked increase which one finds in normal individuals. The experiments also demonstrate the altered vascular responses of the sympathectomized dog, otherwise carbon dioxide as administered in these experiments would have produced a definite leucocytosis. The result is somewhat surprising in the face of the well known local effects of carbon dioxide on capillaries, but in this animal there was no observable change in the pallor or temperature of the ears and no visible evidence of peripheral vasodilatation.

When the sympathectomized animal was placed in a water bath between 40° and 45°C. for a period of fifteen minutes, the ears showed a definite

TABLE 2

Erythrocytes per cubic millimeter of blood obtained from a sympathectomized dog

Exercise does not affect the count

	ACTIVITY LEVEL	HALF-HOUR REST	SEVERE EXERCISE
1	6,000,000	5,800,000	5,980,000
2	6,550,000	6,400,000	6,620,000
3	6,750,000	6,680,000	6,580,000
4	6,680,000	6,500,000	6,600,000
5	6,420,000	6,480,000	6,550,000
6	6,700,000	6,580,000	6,620,000
7	6,560,000	6,400,000	6,520,000
8	6,740,000	6,680,000	6,700,000
9	6,600,000	6,680,000	6,720,000
10	6,750,000	6,720,000	6,780,000

pink color but in spite of this peripheral effect and in spite of the fact that the animal panted vigorously and showed evidence of muscular rigidity due to the development of alkalosis the leucocyte count rose only to 10,800 which is the activity level. Normal dogs show a striking leucocytosis under these conditions. The effects of heat therefore are in complete accord with those of carbon dioxide and indicate the difficulty of altering the vascular system in such a way as to induce leucocytosis. It may be added that carbon dioxide did not alter the number of erythrocytes.

A study of the number of erythrocytes was conducted as a routine at the time all leucocyte counts were made. These counts are presented in table 2 which shows that there is no change in red blood cells, due to changes from rest to exercise. Since exercise normally raises the dog's erythrocyte count and since this increase is attributed to contraction of the spleen under the influence of the sympathetic (and adrenal) system, the obvious conclusion is that there is no contraction of the spleen due to exercise or to

carbon dioxide in the sympathectomized dog. This confirms the findings of Izquierdo and Cannon (7).

In the work reported above the leucocyte count has been used as an index of vascular lability, or stability. The relative constancy of the count after sympathectomy under conditions which normally cause marked leucocytic fluctuations speaks definitely for a static state of the vascular bed. The facile readjustments and reciprocal interplay of the different parts of the vascular system which are normally responsible for leucocytic variations apparently do not take place under the conditions of ordinary voluntary activity of the sympathectomized dog. The conception of a static state does not, however, imply that the blood vessels are unable to respond to dilator influences under other and suitable conditions.[1]

SUMMARY

1. Vascular tone develops and is maintained after complete sympathectomy. Procedures which normally cause peripheral vasodilatation apparently had little effect and the vascular changes which ordinarily produce variations in the leucocyte count did not produce fluctuations comparable to those in normal animals.

2. The activity level of the leucocyte count after sympathectomy was distinctly lower than normal, while the resting, or basal level was higher than normal.

3. Neither severe exercise, carbon dioxide nor heat caused the leucocytosis found in normal dogs or in man.

4. There was little change in the differential count due to exercise.

5. The erythrocyte count did not vary from rest to activity.

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[1] In a personal communication Professor Cannon informs us that such dilatation has been demonstrated in his laboratory. Since this manuscript left our hands Bradford Cannon (8) has reported that after sympathectomy the blood pressure of dogs returns to and remains normal.

BLOCK OF THE SPINAL CORD PRODUCED BY COLD

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Received for publication June 18, 1931

Muscular reflexes in the spinal animal differ from those seen in the decerebrate preparation in respect to threshold of stimulation, the extent of after-discharge, and other characteristics (Forbes, 1922; Fulton, 1926). These differences are ascribed to the removal of the influence of certain centers in the medulla which are responsible for decerebrate rigidity, and perhaps to the elimination of so-called long-circuiting pathways. Attempts have been made to elucidate these influences by comparing the reflex responses before and after surgical transection of the spinal cord. This method has yielded a considerable amount of valuable information (Forbes, Cobb and Cattell, 1923; Forbes and Baird, 1929), but it is impossible to follow the transition from one state to the other satisfactorily by this method on account of the extensive stimulation of the animal resulting from surgical transection itself. It usually requires two or three minutes for the muscular spasms and convulsions to subside, and even after the animal has quieted down, the question remains as to whether the differences noted during the period immediately following are due entirely to removal of the influences of the upper centers or pathways, or whether they depend in part upon the excitation induced by the operation. We have therefore developed a method for producing a functional block in the spinal cord without excitation, by locally cooling the spinal cord at the level of the last rib. The block so produced is reversible, and thus offers further advantages for the study of the influence of higher centers upon those in the lower portion of the spinal cord.

Special apparatus. The essential feature of method consists in passing a stream of cold water through a flattened copper tube which has been inserted beneath the spinal cord of the animal at the level at which it is desired to produce a functional block. The details of this tube and the clamp by means of which it is held in place are shown in figure 1. The tube itself is made of copper, annealed to make it as pliable as possible. In order to bend it into the shape illustrated without collapsing the walls, it was filled with rosin during the bending process, the rosin later being melted out. Even with this precaution the shaping requires considerable care to prevent cracking and buckling. The particular shape illustrated makes it

easy to slip the tube gently beneath the spinal cord without undue displacement of the latter.

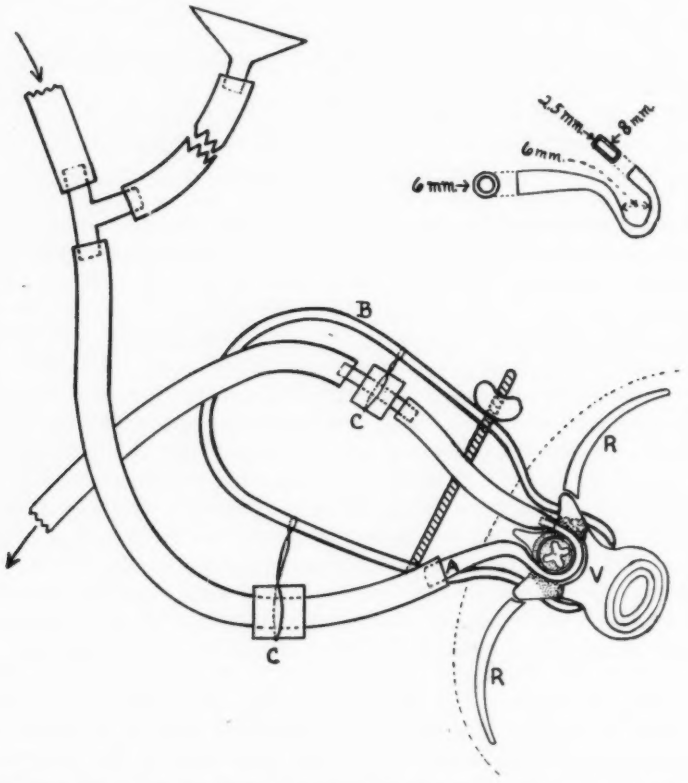

Fig. 1. Details of cooling tube, clamp and method of application to spinal cord. *A*, cooling tube, shown in detail in upper right-hand corner, fashioned from annealed copper tubing 6 mm. outside diameter, 4.5 mm. inside diameter. *B*, clamp, with teeth engaging the body of the 13th thoracic vertebra. *C*, corks, attached to double copper wires soldered to clamp. *R*, 13th ribs. *V*, 1st lumbar vertebra seen in section looking cranially. Dotted area on transverse processes indicates extent of notches cut to admit cooling tube. The spinous process of this vertebra has been removed. The one shown in diagram belongs to the 12th vertebra. Spinal cord is shown in place in the loop of the cooling tube. Inflow and outflow indicated by arrows. Funnel allows introduction of warm water.

The clamp which supports this tube is made from a strip of brass 16 mm. wide, narrowing at its jaws to 12 mm. This width permits it to slip easily between the transverse processes of consecutive vertebrae, and the

teeth in its ends bite into the bodies of the vertebrae when the thumb screw is tightened. Rubber tubes which connect with the copper tube pass through pieces of cork which are attached by means of two strands of copper wire to the clamp. This arrangement allows a flexibility of adjustment of the tube to individual animals.

Ice water flows by gravity from an earthenware crock through heavy-walled rubber tubing to the cooling device. Its temperature is usually about 2°C. on entering the cooling tube and approximately 6°C. at the end of the outflow tube. A glass funnel connected with the inflow tube by means of a T joint allows the rapid introduction of warm water in place of the cold when it is desired to restore the function of the cord. The flow of fluid is readily controlled by means of ordinary tubing clamps.

METHOD. The cat is anesthetized with ether, a tracheal cannula is inserted and carotid arteries tied. The spinal column is then exposed at the level of the last rib. The musculature over the first and second vertebrae is then separated from the vertebral column by blunt dissection and laminectomy performed with narrow-billed rongeur forceps. It was found advantageous to begin the exposure by first removing the caudal articular processes. This exposes the cord and admits the jaws of the rongeur forceps. After exposing the spinal cord to view, the medullary canal is widened to admit the cooling tube by cutting a notch down each side of the medullary canal into the body of the first lumbar vertebra just caudal to its transverse processes, continuing down to a depth corresponding to the bottom of the medullary canal. Before cutting these notches, it is essential to clean away all tissue by blunt dissection from the area to be cut, in order to avoid hemorrhage from the small vessels lying on the periosteum.

The cooling tube, with inlet tube attached, is then pushed under and around the cord without raising the cord more than the actual thickness of the tube itself, about 2.5 mm. With careful manipulation this need cause no disturbance to the animal. While holding the cooling tube firmly against the vertebra, the retaining clamp can then be put in place just anterior to the transverse processes of the first lumbar vertebra and the outlet tube snapped over the free end of the cooling tube. The inlet and outlet tubes are made of thin-walled flexible rubber tubing but are stiff enough to hold the cooling tube firmly against the vertebra, thus preventing traction on the spinal cord. The wound is then closed and the animal decerebrated at the level of the anterior colliculi by means of the Sherrington guillotine. We then partially immobilize the right hind leg by cutting the psoas muscles in the groin and the nerves to the hamstring muscles. The anterior crural nerve is also cut except for the saphenous branch, to which tube electrodes (cf. Forbes, Davis and Lambert, 1930) are applied for stimulation. The limb is then fixed by means of a femur clamp and by a drill through the lower third of the tibia. Flexion of the right ankle joint

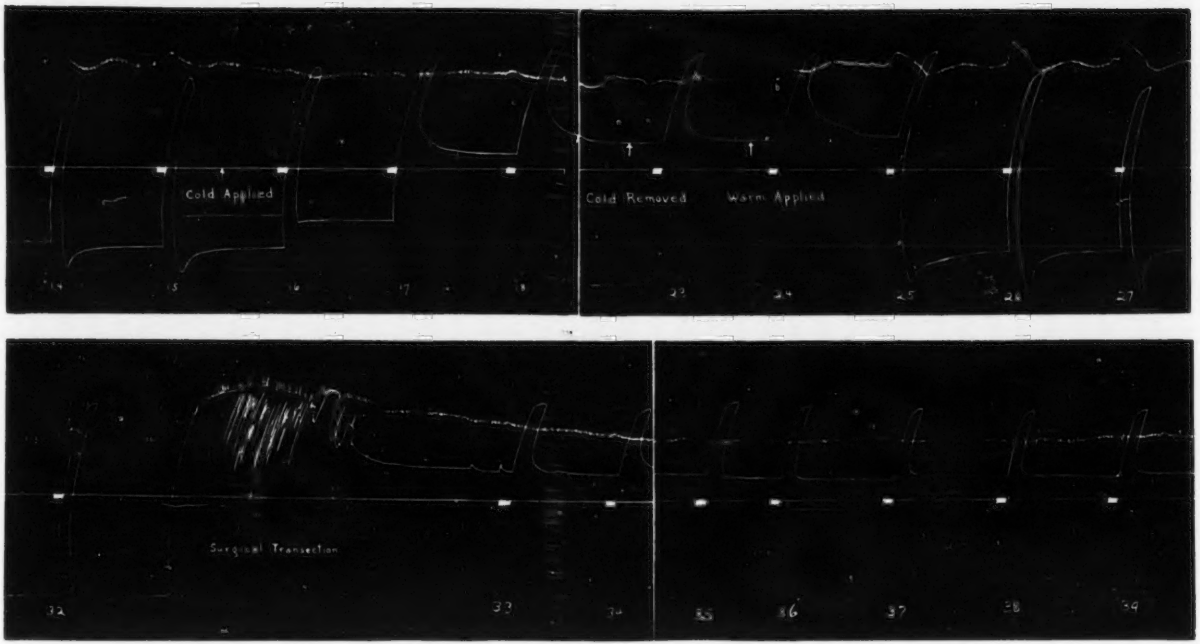

Fig. 2. Record of flexion reflexes and blood pressure, experiment 4, 4/4/31. Numbers indicate periods of stimulation, at minute intervals, from beginning of experiment. *14* and *15* are typical of the initial series. Arrows show beginning and end of flow of cold water and beginning of flow of warm water. The effects of surgical transection, between *32* and *33*, are typical. Characteristic "spinal" responses are shown from *33* on. At *b* (stim. *24*) a clot spontaneously dislodged from the carotid cannula and allowed a sudden increase in the amplitude of the pulse record. The occasional waves in the blood pressure record between *34* and the end are associated with gasping respiration. The signal line is also the line of zero blood pressure.

may then be recorded on a smoked drum by means of a muscle lever working against the light traction of an elastic band. The contractions thus recorded are essentially isotonic. The resting position of the lever between stimulations serves as a crude index of the degree of extensor tone. On the same drum may be recorded the carotid blood pressure and also the moment and duration of stimulation.

It was found most desirable to place the animal upon an electric heating pad, as even with this precaution the animals sometimes gave evidence of chilling during passage of cold water around the spinal cord.

TABLE 1

The figures for blood pressure refer to the general level at which it stood between periods of stimulation and not to the rise in pressure which follows stimulation when the cord is not blocked or cut. The low level at the end of experiment 4 may be due in part to a rather considerable loss of blood incurred during the removal of a clot from the cannula just before transection.

In estimating muscular tone the level of the writing point during the initial control period was arbitrarily taken as zero. Negative values represent a loss of extensor tone. The degree of decerebrate rigidity estimated on the basis of resistance to passive flexion varied considerably in these preparations. Quantitative comparisons of one experiment with another with respect to muscular tone are without significance.

EXPERIMENT	BEFORE COOLING	DURING COOLING	AFTER WARMING	AFTER SURGICAL TRANSECTION
Blood pressure in millimeters of mercury				
1	80	74	84	50
2	110	90	100	76
3	60	50	70	52
4	90	76	90	60
Extensor tone in millimeters above or below initial level				
1	0	−14	−10	−25
2	0	−22	+8	−20
3	0	−16	−5	−21
4	0	−47	+10	−45

EXPERIMENTAL RESULTS. Submaximal tetanizing stimuli were delivered from a Harvard inductorium with iron core in place at a rate of about 50 break shocks per second. The animal was stimulated regularly for a period of five seconds every minute throughout the experiment, except during the reflex spasms consequent on cutting the cord at the end of the experiment. A series of normal responses was first recorded. Care was taken to continue this preliminary series long enough to allow both the vascular and the flexion reflex responses to attain an approximately steady state. Ice water

was then allowed to flow through the cooling system at a rate of about 130 cc. per minute. After a steady state had again been reached, the cord was again warmed by passing about 100 cc. of water at a temperature of about 38° to 40°C. around the spinal cord. As shown in figure 2 and in table 1, the muscular tone, which was considerably diminished consequent on cooling the cord, and the vascular responses which had been practically abolished, both returned to practically the normal level. Likewise the movements of the foot, which usually showed the characteristic alteration illustrated in the figure, returned to the normal decerebrate pattern. The cord was then transected surgically with scissors, and after the resulting spasms had subsided and blood pressure had returned to the spinal level, stimulation was again resumed. The animals were regularly in good condition at this stage of the experiments, except for the low blood pressure which is characteristic of the spinal animal.

We do not wish to attach any great significance to the quantitative changes in the flexion reflex response as recorded by this relatively crude technique. Further analysis of these changes is contemplated in the near future, employing isometric recording supplemented by galvanometric studies. Certain comparisons are, however, significant as indicating the attainment of a reversible functional block of the spinal cord.

The first comparison deals with the normal decerebrate state and the condition of the animal after the cord has been cooled and again warmed. The similarity indicates that whatever change is produced by the cold, at least during the relatively short time (5 to 10 minutes) during which it was maintained in these experiments, is readily and completely reversible. The similarity in vascular and muscular responses is illustrated in the figure. Columns 1 and 3 of table 1 indicate that the general level of blood pressure between stimulations returns to essentially the same level, sometimes a little higher, sometimes a little lower. The general level of muscular tone is indicated in the same columns, and the same average agreement is evident. Although individual preparations varied somewhat in their behavior, the fluctuations seem to be of minor importance as compared with the general trend of the changes observed.

The second set of comparisons deals with the condition of the animal during the period when the cord was cooled as compared with the period after surgical transection. The similarity which is evident here indicates that a complete functional block was actually produced by the cooling. Not only does the blood pressure fall to the characteristic low level but the character of the vascular reflex is the same under both conditions. The barely perceptible rise in the blood pressure is that which might be expected from local reflexes governed by the lower part of the spinal cord. No respiratory reflexes in response to the sensory stimulation were seen during the cooling periods. The level of muscular tone falls during the application

of cold to as great, or nearly as great, a degree as following transection, indicating the practically complete abolition of decerebrate rigidity.

Of particular practical interest is the nature of the response of the animal when cold is first applied to the spinal cord. In all of our experiments the level of blood pressure fell off smoothly, and no disturbance of the neuromuscular system could be detected. When the effects of cold became established, usually within one minute, the flexion reflex jumped to a higher level than normal, and a marked decrease of extensor tone occurred. In contrast to this quiet transition is the violent storm of muscular activity which attends surgical transection. Figure 2 shows a typical reaction. The subsequent warming of the spinal cord was accompanied by the same smooth transition that attended the application of cold.

We are indebted to Miss Elizabeth Lambert for valuable assistance throughout these experiments, and to Dr. Frederick B. Skinner for assistance during the preliminary tests.

SUMMARY

1. A method is described for locally cooling the spinal cord in the decerebrate cat.

2. It is shown that such local cooling produces a functional block in the cord, and that the resulting condition of the animal corresponds closely with that developing after surgical transection.

3. The production of such a functional block is not attended by the usual excitatory effects in the form of muscular spasms and high blood pressure, which inevitably follow immediately upon surgical transection. The utility for future research of avoiding these excitatory effects is pointed out.

4. The functional block produced by cold is readily and completely reversible up to intervals at least as long as 10 minutes.

THE NATURE OF THE SUBSTANCE CAUSING OVULATION IN THE RABBIT[1]

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Received for publication June 22, 1931

Friedman (1) has shown that ovulation can be induced in the rabbit by a single intravenous injection of the urine of pregnant women. Bellerby (2), Hill and Parkes (3) and Jares (4) obtained the same results by the intravenous injections of anterior lobe extracts. It has been quite clearly demonstrated by Friedman (5) that a hormonal mechanism is involved in inducing ovulation in this animal, and the work of Fee and Parkes (6) showed that normally, the intact hypophysis is necessary for ovulation to occur following copulation. Later, Hill and Parkes (7) discovered that it was very difficult to induce ovulation with pregnant human urine in hypophysectomized rabbits while such was not the case when active anterior lobe extracts were used. This difference in action led these workers to believe that the pregnant urine was different from anterior lobe extracts.

The evidence to date on the hormonal initiation of the ovulation reaction in the rabbit points to the anterior lobe as the origin of the causative agent. This paper presents some results of using different anterior pituitary materials to induce ovulation in the rabbit in an attempt to determine which of the known hormones of this gland is the stimulating factor. The preparations used were also checked for other physiological reactions attributed to this gland, namely, body growth, follicular growth, and lutein development in the white rat.

GENERAL METHODS AND PROCEDURE. The rabbit, unlike the rat, does not have a vaginal reaction indicative of the state of the oestrous cycle. Kunde and Proud (8) have shown that copulation will take place irrespective of the vaginal picture. Martin and Fabias (9) have shown that it is possible to elicit ovulation in the rabbit during pregnancy. The use of pregnant rabbits would complicate the problem under discussion so that such animals were not used in these experiments. The procedure followed here was to isolate sexually mature female rabbits for a period of 10

[1] This work was assisted in part by grants from the Committee for Research on Problems of Sex of the National Research Council administered by Dr. Frederick L. Hisaw.

to 20 days at which time they were almost always in heat. It was observed that the vulva becomes exceedingly purple and swollen, especially in the gray chinchilla variety, at the time the follicles are in the proper physiological condition for ovulation and that such females would invariably accept the male. Fifty-five rabbits were used in these experiments.

Injections were usually made about four o'clock in the afternoon so that by the following morning an exploratory operation could be performed for diagnosis. The materials to be tested were injected into the marginal ear vein, the rabbits being able to tolerate as much as 10 ccm. if administered slowly. If ovulation takes place it can readily be detected grossly or with the aid of a hand lens, as the follicle appears pointed and usually a small clot is found in the ruptured wall. Whenever there was cause to doubt that ovulation had taken place, the ovary was removed and that particular part sectioned (fig. 4). All the follicles usually do not rupture, a condition which is quite normal, even after copulation. The finding of a single ovulation point, however, was considered positive though these instances were quite rare. No particular attention was paid to the time that elapsed between the injection and the diagnosis. It has been established that approximately the same time elapses between initiation of ovulation by copulation or by injections of stimulating substances, with perhaps a slightly longer latent period in favor of the injections. Usually operations were made from fifteen to twenty hours following injections.

The various materials tested were the urines of pregnant women, castrate women and pregnant cows; growth hormone prepared by Van Dyke[2] (10); and follicular stimulating and luteinizing fractions of sheep pituitary prepared in this laboratory.[3] The materials used for stimulating ovulation were also tested on sexually immature rats for the sex hormones of the anterior lobe. The hormones were injected twice daily for five days and the animals killed at the end of the fifth day. The ovaries of the immature rats were usually weighed to obtain an indication of the degree of development for comparison of the different preparations, and also sectioned to study more accurately the type of cellular change produced by the several hormones. Over one hundred rats were used in these experiments.

EXPERIMENTS AND RESULTS. *Urine of pregnant women.* The four reactions attributed to the anterior pituitary substances which are concerned in this work are body growth, follicular growth, and ovulation and luteinization of the follicle. The urine of pregnant women will elicit

[2] The growth hormone was prepared in Doctor Van Dyke's laboratory and with his kind permission was made available for use here.

[3] The sheep pituitary was kindly furnished by Parke, Davis and Co.

ovulation in the rabbit, yet according to the careful work of Evans and Simpson (11) and others, such urine contains none of the growth hormone. Since the urine of pregnant women has been shown to contain the follicular stimulating and the luteinizing hormones through the researches of Zondek (12), it would seem that the *growth inducing hormone, since it is not present in the urine, is not the one which promotes ovulation in the rabbit.* The remaining possibilities of the known hormones are the follicular stimulating and the luteinizing hormones or possibly an unknown substance.

The results in table 1 confirm the work of Friedman that the urine of pregnant women will induce ovulation in the rabbit. It was found that

TABLE 1

Ovulation with urine of pregnant women

RABBIT	AMOUNT	OVULATION	REMARKS
	cc.		
RA 49	5	Positive	Pregnant 8 months
RA 52	5	Positive	Pregnant 8 months
RA 91	5	Positive	Pregnant 5 months
RA 92	5	Positive	Pregnant 5 months
RA 96	5	Positive	Pregnant 5 months
RA 89	4	Positive	Pregnant 5 months
RA 107	2	Positive	Pregnant 5 months 1 ovulation point
RA 90	1	Negative	Pregnant 5 months
RA 108	1	Negative	Pregnant 5 months
RA 121	1	Negative	Pregnant 5 months
RA 122	1	Negative	Pregnant 5 months
RA 95	5	Positive	Pregnant 5 months boiled 8 minutes
RA 53	5	Positive	Pregnant 8 months boiled 15 minutes
RA 97	5	Negative	Pregnant 5 months boiled 60 minutes
RA 71	2	Positive	Prolan B = 20 cc. urine
RA 72	2	Positive	Prolan B = 20 cc. urine
RA 74	2	Negative	Prolan B = 20 cc. urine boiled 15 minutes

1 cc. of urine of a five months' pregnancy case would not induce ovulation but 2 cc. would. Five cubic centimeters seemed to be the optimum amount since this is readily tolerated by the animal and almost invariably insures a positive reaction. However, when 1 cc. of this same urine was injected over a period of five days into a sexually immature rat it was found to be sufficient to give a positive Zondek test for pregnancy with the formation of one to several corpora lutea (fig. 2). The importance of this fact will be brought out later in comparing these results with those obtained for urines of castrate women.

Zondek reports that boiling will destroy the active material in pregnant urine which is responsible for growth of the ovaries of sexually immature

rats and mice. Table 1 shows that prolonged heating at 100°C. will also destroy the substance responsible for ovulation.

The follicular stimulating and luteinizing substances in pregnant urine can be concentrated by a method devised by Zondek (13) wherein it is freed from follicular hormone and toxic materials. Briefly, this consists in precipitating the active substance with five volumes of 95 per cent alcohol, washing the precipitate with ethyl ether, and redissolving the active material in a definite volume of water. This preparation was spoken of by Zondek as Prolan B. and was tested only on rats and mice. This method can also be used to concentrate the ovulating principle from urine as seen in table 1.

Growth hormone. By deductive reasoning from the experiments reported on pregnant urine, the growth hormone was eliminated. However, it was thought that a negative test from an active growth hormone would be more conclusive evidence that this substance was not the ovulating agent. An active preparation of the growth hormone obtained from Van Dyke and Wallen-Lawrence was used for this purpose. Their method of preparation of this hormone, briefly, is to grind up fresh beef pituitaries with dilute NaOH and let stand over night at 0°C., filter through glass wool, neutralize to pH 7.2, precipitate the active material with 20 per cent Na_2SO_4, and take up the active material in a definite quantity of distilled water. The active preparation is sterilized by use of a Berkefeld filter and is potent for restoring normal growth in hypophysectomized adult rats. This preparation was found by these workers to be devoid of the sex influencing hormones of the anterior lobe since it did not interfere with the normal cycle of adult female rats.

As a check on the test for the detection of the sex stimulating hormones in this growth hormone preparation, it was injected into sexually immature rats. This material failed to induce precocious maturity in such animals. Van Dyke has also found the same thing to be true (personal communication); hence it seems that the sex stimulating hormones are not present in appreciable quantities. But when this same extract was injected into rabbits in oestrum, *ovulation always resulted.* The amount required was 0.5 cc., which is less than a detectable amount for growth. Heat also will destroy the active ovulating principle in this growth hormone preparation.

The results from the growth hormone and the pregnant urine would superficially seem to indicate that some unknown substance was producing the reaction. The fact that the growth hormone did not affect the ovaries of immature rats may not be a true characterization of the potentialities of Van Dyke's extract, but the amounts used were certainly sufficient to cause a marked increase in body growth. Evans (14) states that his growth hormone prevents normal sexual maturity in young rats yet will

luteinize the ovaries of adult females. The difference between these two preparations may be one of purity, and the probability may be suggested that the extract made by Evans contained the luteinizing principle in addition to the growth hormone. It is also possible that Van Dyke's hormone may mask or inhibit the action of either the follicular stimulator or the luteinizer which would result in negative effects on immature rats. That this growth hormone is actually antagonistic to or inhibits the follicular stimulator may be concluded from the results of the following experiment.

Sexually immature rats received simultaneous injections of a follicular stimulating hormone prepared by the method of Fevold, Hisaw and Leonard (15) and the growth hormone of Van Dyke. Table 2 shows that the growth hormone inhibits or in some way prevents the action of the potent follicular stimulating hormone. Whether or not it inhibits the action of the luteinizing hormone is very hard to determine since the latter has been found to be detectable only after the ovary has been acted upon by the follicular stimulating hormone (15). Van Dyke reports that his preparation has no effect on the oestrous cycle of female rats so that one may be reasonably safe in saying that his hormone cannot contain a great deal of the sex influencing hormones of the anterior lobe. All these facts leave much doubt as to which of the known anterior lobe hormones are active in producing ovulation.

Prolan A. Zondek (13) has shown that the urines of castrate women and women who have passed the menopause are potent for a substance that produces only follicular development in the ovaries of immature rats and mice. Later he (Zondek, 12) stated that a small amount of lutein tissue can be found in the ovaries of immature rats injected with such urine preparations when large amounts are used. These results were confirmed in this laboratory though many failures in obtaining a source of potent Prolan A were encountered. Even when present, the follicular stimulating substance is not found in all such urines in quantities that are readily detectable; the most potent sources contain detectable quantities only in amounts of urine equal to 10 to 20 cc. An immature rat cannot tolerate such quantities of urine so as a consequence the Zondek concentration method as previously described was employed in preparing extracts of Prolan A.

The results obtained from an active preparation of Prolan A are indeed astounding in that the ovaries may be increased in weight as much as 1000 per cent and contain only follicles when an extract equivalent to 50 cc. of urine is injected. The occasional luteinization of the sexually immature ovary produced by Prolan A is extremely slight and always very much less than the amount of luteinization produced by 1 cc. of pregnant urine where fully formed corpora lutea are always present. On

the other hand, Prolan A equivalent to 50 cc. of urine is at least 50 times stronger for the production of follicular growth than 1 cc. or even 5 cc. of pregnant urine, and 1 cc. of pregnant urine is at least 50 times stronger than the amount of Prolan A equal to 50 cc. of urine for the production of corpora lutea. However, *Prolan A equal to 50 cc. of urine was capable of provoking ovulation while 1 cc. of pregnant urine was not capable of doing so.* From this it would seem that the ability to produce ovulation is not correlated with the ability to produce luteinization. At the same time it does not seem that the follicular stimulator is responsible for ovula-

TABLE 2

Effect of growth hormone and extracts of cow urine on the follicular stimulator from sheep pituitary

RAT	FOLLICULAR STIMULATOR DOSE—0.1 GR.	DAILY INJECTION	VAGINA OPEN	WEIGHT OF OVARIES	CONDITION OF OVARIES
AL507				*grams*	
a	0.5 cc. daily	Pregnant cow, urine 0.8 cc.	3 days	0.0635	Follicles and corpora lutea
b	0.5 cc. daily	Pregnant cow, urine 0.8 cc.	3 days	0.0507	Follicles
c	0.5 cc. daily	Normal cow, urine 0.8 cc.	3 days	0.0507	Follicles and corpora lutea
d	0.5 cc. daily	Normal cow, urine 0.8 cc.	3 days	0.0583	Follicles
e	0.5 cc. daily	Growth horm. 0.8 cc.	5 days	0.0144	Immat. 1 corpora lutea
f	0.5 cc. daily	Growth horm. 0.8 cc.	Closed	0.0138	Immature
AL548		Growth horm.			
a	0.5 cc. daily	0.8 cc.	Closed	0.0103	Follicular stimulator weak but sufficient to act on control in 5 days
b	0.5 cc. daily	0.6 cc.	Closed	0.0107	
c	0.5 cc. daily	0.4 cc.	Closed	0.0115	
d		0.6 cc.	Closed	0.0100	
e	0.5 cc. daily		5 days	0.0120	

tion because the minimal amount of Prolan A required to induce ovulation is many times the amount of Prolan A which is apparently contained in the dosage of pregnant urine to produce ovulation.

Many negative results were obtained in testing various urine extracts which emphasizes the difficulty in selecting individuals who are potent sources of the follicular stimulating hormone. The best extract was obtained from a 24 hour sample of 600 cc. of urine from a woman who had been castrated 10 years previously. The results reported for rats were based on this sample as well as several of the positive cases of ovulation.

A 24 hour sample of urine equal to 1700 cc. taken from the same individual the next day was found to be practically inactive for rats in doses equal to 25 cc. of urine. Very little is known as to the reason for the individual and daily variation in hormone output.

Pregnant cow urine. It has been shown by Zondek (16) that the urine of pregnant cows concentrated ten times, would not give the pregnancy test in immature rats as shown by the production of follicles and corpora

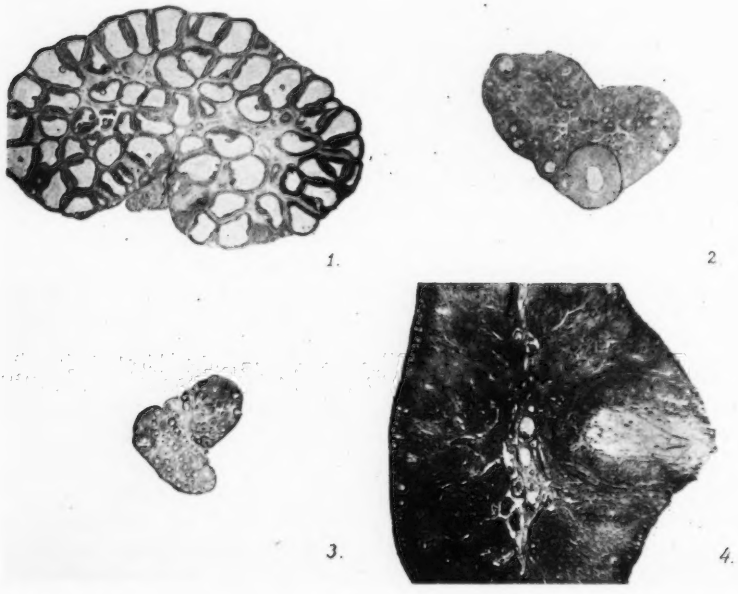

Fig. 1. Ovary of immature rat injected with Prolan A equal to 50 cc. of urine. Note the absence of lutein tissue.

Fig. 2. Ovary of immature rat injected with 1 cc. of urine of a 5 months pregnant woman. Note the well developed corpus luteum.

Fig. 3. Ovary of immature rat.

Fig. 4. Section of ruptured follicle in rabbits ovary induced by the intravenous injection of Prolan A.

lutea. This suggested the possibility of testing the urine of pregnant cows, which is known not to contain any anterior lobe hormones, to see if it would promote ovulation in the rabbit. The results of three experiments show that similar preparations of pregnant cow urine will not produce ovulation in the rabbit. Thus the pregnant cow may be added to the list of animals whose urines do not contain anterior lobe hormones or are capable of eliciting ovulation.

Extracts of anterior lobe. It has been possible to prepare two potent fractions from an aqueous pyridine extract of the anterior lobe of the sheep in accordance with methods developed in this laboratory. One of these which is water soluble, produces primarily follicular development in the ovaries of sexually immature rats, and the other which is water insoluble produces no follicular effect on such ovaries. However, when the two fractions are united, a typical luteinized ovary results, which indicates that the water insoluble fraction is potent for luteinization when the follicles have been stimulated by the follicular stimulating hormone. By injecting either of these two fractions intravenously into rabbits, it was found that *both were equally as potent in producing ovulation in doses equivalent to 0.1 gram.* Although the minimal effective dose for ovulation has not been determined in either fraction, it should be pointed out again that one fraction has the ability of producing follicular growth and the other one does not and yet again there does not seem to be any correlation between this fact and the ability to produce ovulation.

Discussion. To assign a specific hormone from the anterior lobe as the stimulating factor for ovulation in the rabbit is treading on dangerous ground in view of the present tendency of accounting for the various reactions by a multiplicity of hormones from this gland. However, an examination of the facts presented may be worthy of consideration as a working hypothesis for the isolation of the active principle. The urine of pregnant women seems to contain a large amount of the ovulating hormone yet it has been definitely shown not to contain the growth principle. The remaining known hormones in such urine are the female sex hormone (oestrin), the follicular stimulating hormone, and the luteinizing hormone. The oestrin can be removed as is done in the preparation of Prolan B and yet the remaining fraction can induce ovulation. So the ovulating principle would be assigned to either the follicular stimulating hormone or the luteinizing hormone or to some unknown factor.

But a wholly different interpretation must be placed on these findings when it is discovered that the growth hormone of Van Dyke is active in producing ovulation yet is free from the sex stimulating hormones for rats. The probability was pointed out that the growth hormone may inhibit or mask the action of the follicular stimulating preparation or the luteinizing preparation, and that if any were present in the original growth hormone it might be masked in the same way. Such possibilities seemed likely from the results obtained by simultaneous injections of the follicular stimulator and the growth hormones into immature rats. It is the interpretation of the author that Van Dyke's preparation contains neither of the sex influencing hormones for the following reasons. First, Van Dyke's growth hormone when injected alone into sexually immature rats does not produce sexual precocity; second, Van Dyke's hormone is different from that of

Evans in that the former will not disturb the cycle of female rats while that of Evans will produce a luteinized ovary; third, if ovulation is due to one of the sex hormones, why does not the antagonism mentioned above hold when Van Dyke's growth hormone is injected intravenously into the rabbit? The evidence then would point quite strongly to the possible existence of an ovulating principle which may be separate from the other known anterior lobe hormones.

A fact which casts doubt on the luteinizing hormone being responsible for ovulation is that the ability of a preparation to luteinize the follicles of rats is not correlated quantitatively with its ability to promote ovulation. Prolan A prepared from the urine of a woman castrated 10 years previously caused predominantly follicular development in the ovaries of immature rats. Even when given in large doses equivalent to 50 cc. of urine, very little if any lutein tissue may be found in the immature ovary. Yet, this amount of Prolan A will cause ovulation in the rabbit. On the other hand, doses of pregnant urine equal to 1 cc. will produce greater luteinizing effects than 50 cc. of urine containing Prolan A, yet will not cause ovulation in the rabbit. A minimal dose of 2 cc. was found necessary in the sample studied to produce a positive result. It is important to note that the minimal amount of pregnant urine to induce ovulation in the rabbit and luteinization of the ovary of the immature rat was from the same 24 hour sample of a 5 months pregnant woman. The absolute amounts may possibly vary from individual to individual but the relative amounts are the important consideration.

A question may arise in the light of the work here and in other laboratories as to whether the anterior lobe-like effects of the urine of pregnant and castrate women are really due to hormones that can be found in the anterior lobe. As far as ovulation in the rabbit is concerned, it appears from the work of Hill and Parkes on hypophysectomized rabbits that the active substance in the urine may not be an anterior lobe hormone. However, such results alone cannot be conclusive on this question since the interpretation may be one of a difference of mechanism of action or of degree of potency of the active material. Recently, Snyder and Wislocki (17) have found that the urines of pregnant cats, dogs, rats, rabbits, pigs, and monkeys would not elicit ovulation in the rabbit. These urines have also been found not to affect the ovaries of immature rats (with the exception of the urine of the monkey). To this list, the urine of pregnant cattle may be added as seen from the results reported here. So far as the urines have been tested physiologically, wherever it is possible to obtain activity for the ovaries of immature rats, it is also possible to induce ovulation with the same preparation.

Whether the anterior lobe-like hormones arise in the placenta or in the anterior lobe itself is not the question, but certainly these substances from

urine as far as now known are chemically and physiologically similar to those found in the anterior lobe (15). For this reason, it is our feeling that the results obtained from potent urines can be compared with the results obtained from anterior lobe extracts for the ovulation reaction as well as for ovarian changes in immature rats. It is also seen from the study of anterior lobe extracts that both principles, the follicular stimulating and the luteinizing hormones are equally as potent in inducing ovulation on 0.1 gram doses. No quantitative studies for ovulation, as yet, have been made on either fraction.

Another question that may be worthy of consideration is whether or not the rabbit is an animal in which a specialized reaction takes place, and that results for ovulation in this species can be applied to animals that ovulate spontaneously. Many of the hormones are specific in their action and properties as for example relaxin, the hormone which is specific for the relaxation of the pelvic ligaments of the guinea pig and so far has been obtained from only limited sources. Even though the ovulation hormone may be similarly specific, it is the opinion of the author that ovulation studies can be made only on such animals whose own pituitaries are so constituted as not to interfere with the experimental procedure. This opinion is based on an experiment on a limited number of immature female rats whose hypophyses are not functioning sufficiently to complicate the picture with regular cycles. These animals were given Prolan A for 5 days until large follicles were produced in the ovaries. Twenty-four hours following a single intraperitoneal injection of 1 cc. of growth hormone or pregnant urine into the animal, ova were found in the upper part of the tubes while no ova were found in the tubes of the controls. The rabbit may normally be like the immature rat that has been stimulated by the follicular stimulating hormone in which the follicles are first built up and the animal's own hypophysis cannot induce spontaneous ovulation. These indications as well as those pointed out by Friedman show that the method of administration of the hormone plays an important rôle in the type of result to be expected. It may be that the method of injection is only duplicating the necessary concentration of the hormones which takes place normally in the adult animal to produce ovulation.

In conclusion it may be said that no one has yet reported an anterior lobe extract which produces only ovulation, although the results reported here seem to show that qualitatively, the substance responsible for ovulation is not associated with any specific one of the three known anterior lobe hormones. Also it seems that the efficacy of a preparation to promote ovulation bears no quantitative relationship to the presence of the previously described active substances. From the interpretation of these results it seems reasonable to suggest the possibility that ovulation may be due to a specific substance.

SUMMARY

1. Urine of pregnant women produces ripe follicles and corpora lutea in the ovaries of immature rats, does not contain the growth hormone, yet will induce ovulation in the rabbit when injected intravenously.

2. Urine of pregnant cattle produces neither follicular nor lutein development and does not cause ovulation.

3. Growth hormone prepared by Van Dyke promotes neither follicular nor lutein development in rats yet does cause ovulation.

4. No quantitative relationship seems to exist between the ability of a hormone preparation to produce follicles and corpora lutea in immature rats and the ability to cause ovulation in the rabbit.

5. Discussion of the reliability of such experiments with the suggestion of a possible working hypothesis for a separate ovulating principle.

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STUDIES ON THE CHEMISTRY AND THE PHYSIOLOGY OF THE PARATHYROID HORMONE

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Received for publication June 18, 1931

In 1928, Burn (1) included in his book on *Methods of Biological Assay* an alternative method for standardizing preparations of the parathyroid hormone. This method is based on a suggestion made by Stewart and Percival (2). Cats are anesthetized with ether and paraldehyde. The hormone is injected intravenously. By this means Stewart and Percival claim to have obtained with ten units a 2 mgm. rise in the serum calcium in two hours. This was the maximum point in their calcium curve. In 1927, Herxheimer (3) reported an even more abrupt rise in the serum calcium in cats. He found a thirty per cent rise in two minutes, with a maximum in ten to twenty minutes. From this it seemed worth while investigating further the response of cats to parathyroid hormone with particular reference to their use in a shorter method of assay.

The chemical and physiological properties of the active principle of the parathyroid glands have been studied by many workers, particularly since 1925, when Collip (4) presented absolute proof that beef parathyroids contained an active principle which was freed by hydrolysis with dilute hydrochloric acid and which prevented or relieved tetany in thyroparathyroidectomized dogs. In the present paper, a brief report will be given of further studies on: a, the effect of rendering the acid hydrolysate alkaline to phenolphthalein in the course of preparing active extracts of the parathyroid hormone; b, the optimum length of hydrolysis and strength of acid; c, the effect of incubation at 35° on the active principle in glands kept in physiological saline at definite pH values; d, the destruction of the hormone by tissue enzymes; e, attempts to further purify the active principle; f, the effect of diet on the degree of response; g, the serum calcium curve obtained by intravenous injection of the hormone in dogs.

EXPERIMENTAL WORK. *Cat method of assay.* Twelve cats were anesthetized with ether and 40 to 100 units of parathyroid hormone were injected intravenously. The serum calciums were followed for the first two hours, during which they showed little or no rise. The complete results are given in table 1.

Effect of rendering the acid hydrolysate alkaline to phenolphthalein. In

the course of preparing their extracts, Collip and Clark (5) noted that when, after acid hydrolysis, the digested mixture was made alkaline to phenolphthalein, most of the residue dissolved. Whether making the solution alkaline had anything to do with the potency of the extract was not shown. This was studied by extracting the glands as outlined by Collip and Clark (5). The fat was removed from the hydrolysate after chilling and the pH adjusted to 5.6. The solution was then concentrated and divided in four equal volumes. The first portion was left unaltered, while the second,

TABLE 1

CAT	WEIGHT	SEX	UNITS GIVEN	CONTROL Ca	Ca OBTAINED AT THE END OF		
	kgm.				10 minutes, 10.05; 45 minutes, 9.91; 1.5 hours 9.86; 2 hours, 10.36		
					10 minutes	1 hour	2 hours
2	4.40	Male	40	10.49	10.30	10.49	10.49
3	4.78	Male	40	10.59	10.49	10.15	10.35
4	2.90	Female	40	10.8	10.3		
5	3.75	Female	80	10.15	10.15	10.1	10.4
6	2.45	Male	40	10.7	10.3	10.6	11.4
7	3.90	Female	60	10.7	10.4	11.25	11.5
8	3.70	Female	90	10.8	10.1	10.9	10.7
9	4.75	Male	100	11.2	10.7	11.2	11.2
10	4.15	Male	100	10.75	10.45	10.5	10.6
11	3.85	Female	100	9.65	9.65	9.6	9.5
12	3.00	Female	100	10.7	10.35	11.25	11.45

TABLE 2

5 cc. EXTRACT NO.	NO. OF DOGS USED	AVERAGE RISE IN Ca PER 100 cc. SERUM
		mgm.
1	8	3.9
2	6	3.3
3	10	3.4
4	6	3.8

third and fourth portions were made alkaline to phenolphthalein and left thus for thirty seconds, thirty minutes, and four hours respectively. At the end of these periods they were readjusted to pH 5.6. Sufficient water was then added to bring each to the same volume. When equal quantities of these extracts were injected in a number of dogs, the average rise in serum calcium obtained for each lot, as shown in table 2, indicated that the alkaline treatment had not altered the potency of the extract.

Optimum length of hydrolysis and strength of acid. Collip and Clark in

preparing extracts, used three or five per cent hydrochloric acid and hydrolyzed from 20 to 60 minutes. In order that any differences obtained might be reasonably assumed due to the strength of acid used and the period of hydrolysis, the procedure followed in the present work was simplified as much as possible, and no attempt made to carry out a purification of the active principle. Equal weights of finely minced glands were digested for either 15, 30, 45, or 60 minutes in 1.5, 3.0 or 5.0 per cent hydrochloric acid. This was carried out both with acetone-preserved glands and with fresh glands which had been packed in "dry ice." At the end of the period of hydrolysis, the mixture was diluted to a litre with hot water, chilled, and the congealed fat removed mechanically. The undissolved residue was centrifuged off and the pH of the solution adjusted to 5.6. This solution was next concentrated in vacuo and any further precipitate

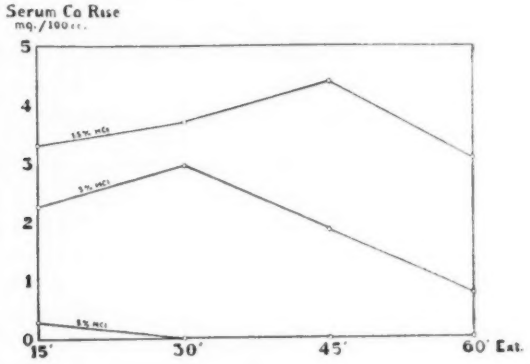

Fig. 1

removed and washed. The filtrate was again concentrated in vacuo and diluted to a definite volume. Five cubic centimeters of each of the twenty-four extracts thus prepared were injected intramuscularly in dogs and the serum calcium elevation at the end of seventeen hours noted. The averages of all the results obtained have been summarized in figure 1.

It is evident from figure 1 that, with 1.5, 3, and 5 per cent hydrochloric acid, the optimum period of hydrolysis is shorter the stronger the acid used, and that hydrolysis with 1.5 per cent acid yields the most potent extract.

Effect of incubation at 35°C. on the active principle of glands kept in physiological saline at definite pH. It is clear from figure 1 that, while digestion of the parathyroid glands with hydrochloric acid frees some of the active principle, too long heating or using too strong acid destroys some if not all

of it. Accordingly, the effect of using acid at a smaller concentration and digestion at a lower temperature for a longer period was investigated.

Four lots of glands, very finely ground, and each weighing 50 grams, were placed in 100 cc. physiological saline in flasks E, F, G and H. Chloro-

TABLE 3

	SAMPLE E		SAMPLE F		SAMPLE G		SAMPLE H	
	pH	0.5 N HCl	pH	N NaOH	pH	N NaOH	pH	N NaOH
Before incubation	6.4		6.8		7.2		7.6	
24 hours at 35°C	6.5		6.8		7.1		7.3	
Correction, cc		0.2				0.1		0.3
Redetermined	6.4				7.2		7.6	
2 days at 35°C	6.4		6.8		7.0		7.4	
Correction, cc						0.2		0.2
Redetermined					7.2		7.7	
3 days at 35°C	6.5		6.8		7.2		7.4	
Correction, cc		0.2						0.15
Redetermined	6.4						7.8	
4 days at 35°C	6.4		6.8		7.2		7.7	
6 days at 35°C	6.6		6.8		7.1		7.5	
Correction, cc		0.3				0.1		0.1
Redetermined	6.4				7.2		7.6	
8 days at 35°C	6.5		6.8		7.2		7.6	
10 days at 35°C	6.5		6.8		7.2		7.5	
Correction, cc		0.2						0.1
Redetermined	6.4						7.6	
13 days at 35°C	6.4		6.8		7.2		7.5	
Correction, cc								0.1
18 days at 35°C	6.4		6.8		7.2		7.6	
25 days at 35°C	6.4		6.8		7.0		7.5	

form and toluene were added to these as preservatives. Such preparations had a pH of 6.8. The contents of flask F were left at pH 6.8, while those of E, G and H were adjusted by adding acid or alkali to pH 6.4, 7.2 and 7.6 respectively. The flasks were then corked and placed in the incubator at 35°. These preparations exhibited a marked buffering action as seen

by their tendency to maintain or return to pH 6.8 during the incubation. This will be made clear by reference to table 3.

At the end of 13, 20, and 25 days, portions of the mixture in E, F, G and H were centrifuged and 5 cc. of the clear liquid injected intramuscularly in dogs. Bloods drawn fifteen hours later showed no elevation in the serum calcium. Further, when extracts were made in the usual way from the gland residues, such extracts exhibited no potency.

Four more lots of glands adjusted to pH 3, 4, 5 and 6, and incubated at 35° for 5, 12 and 19 days, also exhibited negative results both for the supernatant liquid and for the residues.

Accordingly, it appeared that the active principle was destroyed during the incubation. To ascertain whether this resulted from too long incubation, shorter periods were tried, namely, 4 and 2 days, and then 10, 5, 3, 2 and 1 hours. Again the extracts prepared both from the supernatant liquid and from the residues were inactive.

Destruction of the hormone by tissue enzymes. Since incubation of gland material in physiological saline at 35° for periods of one hour to twenty-five days, and at pH values ranging from 3 to 7.6, failed to yield any of the active principle either in the saline or in the gland residue, it was thought that tissue enzymes might be responsible for the absence of any potency. To test this, the enzymes were destroyed by adding saline at 100° to a fresh lot of glands and, after keeping the temperature above 80° for five minutes, the mixture was cooled to 35°, adjusted to pH 6, and incubated for eleven days. Samples of the solution removed on the fourth and the eleventh days showed no activity, but the residues when extracted in the usual way at 100° with 1.5 per cent hydrochloric acid yielded an active extract. Attempts to extract the active principle by heating at 100° in a physiological saline solution did not prove successful. From this it was evident that heating is necessary to destroy tissue enzymes which would otherwise inactivate the active principle, and that acid is necessary to free the active principle from the glands. That the temperature of the incubator was not responsible for the destruction of the active principle was seen from the fact that a sterile tube of "Parathormone" lost none of its potency when incubated at 35° for thirty days.

Attempts further to purify the active principle. From the chemical similarity of the active principle to insulin, it seemed reasonable to suppose that the methods used to purify insulin might also prove helpful in attempting to purify the active principle of the parathyroid glands. In 1925, Collip (6) applied Abel's insulin method (7) using brucine acetate and pyridine, but obtained no success. In 1929, Harington, Scott, Marks and Trevan (8) reported another successful method for purifying insulin by using saponin. The saponin appeared to lower the surface tension, to inhibit precipitation in general and to sharpen the isoelectric point. All

saponins were not found equally suitable for this purpose. These authors set up certain specifications which the saponin must meet if it is to be of use. After obtaining a saponin which met these requirements, Harington's method was applied in an effort to obtain a purer fraction from Collip's isoelectric precipitate. The saponin appeared to exert a buffering action for precipitation was gradual. However, all the precipitates obtained were not only amorphous but failed to contain any potency. Evidently the saponin method for purifying insulin does not lend itself to the purification of the active principle of the parathyroid glands.

An attempt to split Collip's isoelectric precipitate of the parathyroid hormone into active and inactive fractions was made by approaching the isoelectric point very gradually. This point was approached slowly from the acid side by using buffers and various concentrations of alcohol up to 95 per cent. In no case, however, was any separation accomplished. The isoelectric point was then approached from the alkaline side by the gradual addition of carbon dioxide both in the presence and in the absence of buffers and with or without heating. In no case was any inactive material separated, and the precipitates obtained were not only active, but contained all the activity when sufficient carbon dioxide was added. It is interesting to note that while the addition of sodium hydroxide or sodium bicarbonate to an acid solution of the active principle brought it down at pH 4.8, subsequent solution in further alkali and precipitation with carbon dioxide brought down the active material at pH 6.8. When the carbon dioxide precipitates were dissolved in alkali and treated with alcohol, fine needle-like crystals were obtained. However, they proved to be inactive and subsequent examination showed them to be crystals of sodium bicarbonate.

From the earlier work of Collip and Clark, the active principle was shown to be soluble to the extent of 0.1 per cent in absolute ethyl alcohol. Considering that this fraction might represent a more active portion, some of the isoelectric precipitate was subjected to continuous extraction for forty-eight hours at reduced pressure with absolute ethyl alcohol. The apparatus used is shown in figure 2. No definite activity was shown by the alcoholic extract. The residue remained potent and analysis showed the nitrogen content had not been altered. Similar extraction of the active material both with normal butyl alcohol and with saturated aqueous butyl alcohol failed to yield an active extract, or alter the nitrogen content of the undissolved residue. From this it seemed that continuous extraction with these alcohols was of no value in trying to split the isoelectric precipitate into simple fractions.

Collip, while working with his placental extract, succeeded in throwing down a large inactive precipitate from his A-P-L fraction by saturating with potassium acid phthalate. When this salt was added to solutions

of the active principle of the parathyroid glands, it was found that, within a suitable pH range, the active principle was precipitated, the completeness of the precipitation depending upon the amount of the salt added. If sufficient phthalate were added, the precipitate was found to contain all the activity.

Effect of diet on the degree of response. It is well known that the degree of response to parathyroid hormone not only varies with the species, but different individuals in the same species may show a marked difference in the degree of response. Dogs have been used in assaying extracts of the hormone because of their sensitiveness and because of the ease with which frequent blood examinations can be made. By using twelve dogs and noting the average serum calcium rise, Collip has obtained a value for the commercial product which agrees remarkably well with the value found by Eli Lilly & Co., who prepare and standardize "Parathormone." When

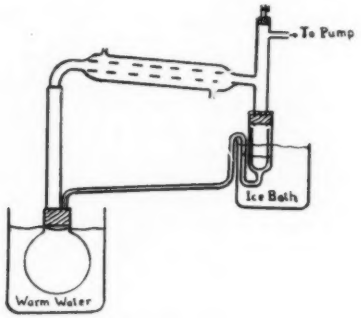

Fig. 2

several extracts have to be standardized at the same time, the number of dogs required may be more than can be readily obtained. This brought up the question whether the same dog will give a constant response. If this could be answered affirmatively, then, by first standardizing a few dogs with an extract of known potency, assays of extracts of unknown potency could be carried out with fewer dogs, thus saving much time and expense, and yet losing nothing in the way of accuracy. To answer this question, four dogs were injected intramuscularly with fifty units "Parathormone" on different occasions and the rise in serum calcium at the end of sixteen hours noted. The results are shown in table 4.

It is evident from these results that the response elicited in the same dog is not constant. It is true that dog 47 showed a remarkably close agreement in the response obtained for the first four injections and dog 48 a fairly constant response for the same occcasions, but, outside of this, the

four dogs referred to in table 4 showed little or no uniformity in the degree of response. Another point of interest brought out in this table is the apparent increased tolerance shown by dogs 47 and 49 with repeated injections, while, on the other hand dogs 48 and 50 tend to show a decreased tolerance. The failure of dog 49 to show any rise in serum calcium with the first injection, while with the second injection the serum calcium rose 4.5 mgm., is worth noting. From the first result one would have been inclined to discard this dog as a non-reactor. Of the sixty-two dogs used in this work, none could be classified as non-reactors, although two of them showed no response to the first injection and several showed less than 0.5 mgm. rise in serum calcium. In this connection it is interesting to note that the response to a second injection has almost invariably been greater

TABLE 4

DATE	DOG 47, 15.7 KGM., FEMALE			DOG 48, 26.2 KGM., MALE			DOG 49, 23.7 KGM., MALE			DOG 50, 17.3 KGM., FEMALE			AVERAGE RISE
	Control	16 hours	Rise	Control	16 hours	Rise	Control	16 hours	Rise	Control	16 hours	Rise	
October 27	10.6	14.8	4.2	11.2	13.3	2.1	11.2	11.2	0	10.9	11.9	1.0	1.8
October 30	10.8	15.3	4.5	11.6	15.1	3.5	11.1	15.6	4.5	10.8	13.6	2.8	3.8
November 3	11.0	15.4	4.4	11.0	13.8	2.8	10.9	14.3	3.4	11.0	13.2	2.2	3.2
November 6	10.6	15.1	4.5	11.0	13.9	2.9	11.8	14.6	2.8	12.1	13.5	1.4	2.9
November 10				10.5	13.9	3.4	10.7	13.3	2.6	11.0	13.5	2.5	2.8
November 27	10.5	14.2	3.7	10.2	16.1	5.9	10.3	12.4	2.1	10.7	14.0	3.3	3.7
December 4	9.9	10.6	0.7	10.6	14.0	3.4	10.2	10.3	0.1	10.4	13.8	3.4	1.9
December 8	9.9	11.2	1.3	10.2	15.4	5.2	11.0	11.2	0.2				2.2
Average	10.5	13.8	3.3	10.8	14.4	3.6	10.9	12.9	2.0	11.0	13.4	2.4	2.8

than that from the first, irrespective of the degree of response from the first.

Inasmuch as many consider a sufficiency of vitamin D essential for the proper functioning of the parathyroid hormone, and since the dogs, cooped as they were in the animal house during the winter months, received little or no sunshine, it was thought that lack of sufficient vitamin D might be responsible for the variation in response obtained. Accordingly, from December 9th to January 12th, vitamin D in the form of cod-liver oil was added to the daily ration of beef heart and rolled oats. However, this did not appear to exert any influence either on the level of the serum calcium or on the degree of response obtained when the hormone was injected.

Since injection of this hormone not only raises the serum calcium, but, according to Greenwald and Gross (9), also increases the amount of calcium excreted, the source of the latter must become smaller with repeated

injections, unless sufficient calcium is supplied in the diet to make up for this loss. Further, inasmuch as the serum calcium starts to rise soon after the injection of the hormone, reaching a maximum in the case of the dog in fifteen hours, when the hormone has been given either intramuscularly or subcutaneously, and in four to eight hours, as will be shown later, if given intravenously, there must be some source of readily available calcium. If the latter has been depleted, the hormone would not be able to exert its full effect, that is, the degree of response may depend not only

TABLE 5

DIET	DATE	DOG 47			DOG 48			DOG 49			DOG 50			AVERAGE RISE
		Control	16 hours	Rise	Control	16 hours	Rise	Control	16 hours	Rise	Control	16 hours	Rise	
Meat	Jan. 15	10.3	12.3	2.0	11.0	15.0	4.0	11.3	11.6	0.3	10.9	12.8	1.9	2.0
	Jan. 19	10.0	11.5	1.5	10.6	15.3	4.7	10.7	11.5	0.8	10.4	11.2	0.8	1.9
Meat and cod-liver oil	Jan. 22	10.1	11.1	1.0	10.9	15.4	4.5	10.7	11.5	0.8	10.5	12.2	1.7	2.0
	Jan. 26	10.0	10.8	0.8	11.0	15.7	4.7	10.7	11.3	0.6	10.5	12.3	1.8	2.0
Meat and Ca lactate	Jan. 29	10.0	10.5	0.5	10.4	16.1	5.7	10.0	11.6	1.6	10.4	12.1	1.7	2.4
Meat	Feb. 2	10.2	10.7	0.5	10.6	14.9	4.3	9.9	10.9	1.0	10.4	11.8	1.4	1.8
Rolled oats	Feb. 5	9.9	10.4	0.5	10.1	13.6	3.5	10.2	10.0	0	10.3	11.1	0.8	1.2
	Feb. 9	10.0	10.5	0.5	10.6	13.0	2.4	10.3	10.3	0	10.5	10.7	0.2	0.8
Rolled oats and Ca	Feb. 12	9.9	10.2	0.3	10.0	13.9	3.9	10.5	10.3	0	10.3	10.7	0.4	1.1
Meat	Feb. 16	10.1	10.5	0.4	10.6	13.4	2.8	10.6	11.0	0.4	10.1	11.5	1.4	1.2
	Feb. 19	10.2	10.9	0.7	10.4	13.9	3.5	10.5	11.0	0.5	10.3	11.5	1.2	1.5
	Feb. 23	10.2	10.9	0.7	10.5	14.0	3.5	10.5	11.0	0.5	10.3	12.0	1.7	1.6
	Mar. 2	10.8	11.9	1.1	10.9	14.6	3.7	10.9	11.0	0.1				1.6

on the strength of the extract used but also on the amount of readily available calcium. If this is true, then the lack of uniformity of response may be due to the variation in the amount of readily available calcium, and an increase in the latter should give a greater and more uniform response. The effect in this direction of adding calcium lactate to the diet was tried but no definite improvement on the uniformity of response was obtained.

Attention was next turned to the cereal in the diet, which was made up of approximately nine parts of rolled oats to one of beef heart. The work of E. Mellanby (10), M. Mellanby (11), of Mirvish and Bosman (12) and

of Mirvish (13) on cereals, with reference to the presence particularly in oatmeal of an antivitamin D factor suggested that the large amount of oatmeal in the diet might be the cause of the irregularity of response obtained.

On January 13th the diet was changed. The dogs were given beef heart only. This was continued to January 20th, when cod-liver oil was added. On the 27th the cod-liver oil was replaced by calcium lactate. On the 30th the lactate was withdrawn. From February 3rd to 10th the dogs were placed on a diet of rolled oats only. On the 10th calcium lactate was added to this oatmeal diet. From February 13th to March 2nd beef heart only was fed. The results obtained in this series of experiments are summarized in table 5.

From these results it is at once evident that dogs on a meat diet gave a more uniform response than when placed on a diet containing either rolled oats or a mixture of rolled oats and meat. The addition of cod-liver oil to the meat diet appeared to improve still further the uniformity of response to parathyroid hormone, while calcium lactate increased the average response slightly. On the rolled oats diet, the response was less. That this was not due merely to an increasing tolerance that would have occurred even on a meat diet is shown by the fact that when the diet was changed back to meat the response gradually rose again.

Before leaving this series of experiments dogs 47 and 49 were injected with 200 units (four times the dose given before) of parathyroid hormone. If the smaller responses elicited by fifty units throughout the latter part of the experiment, as shown in table 5, were due to an acquired tolerance rather than a depleted source of readily available calcium, then the two hundred units should give a response of the order of four times that obtained with fifty units. On the other hand, if it were due to a depletion of the readily available calcium, the response would be of a much smaller order. The values obtained were:

DOG	SERUM CALCIUM		
	Control	16 hours	Rise
47	10.8	15.3	4.5
49	10.6	13.3	2.7

These values point to an acquired tolerance rather than a depletion of readily available calcium as the chief factor responsible for the decrease in response.

While Mirvish (13) prepared an extract from oatmeal which lowered the serum calcium in rabbits as much as 30 per cent in twenty-four to forty-eight hours, oatmeal extracts prepared according to his procedure failed

to produce any definite evidence of lowering the serum calcium in dogs or interfering with the action of "Parathormone."

Serum calcium curves obtained by intravenous injection of the hormone in dogs. While working with Mirvish's oatmeal extract, it was found that the response elicited with "Parathormone" was less if the hormone was given intravenously rather than intramuscularly or subcutaneously. As very few figures were available on the calcium curves resulting from intravenous injection of the hormone, further information was sought as to the shape of these curves. It was found that the maximum point on the curve was reached in four to eight hours rather than in fifteen to twenty-four hours as has been established for intramuscular and subcutaneous

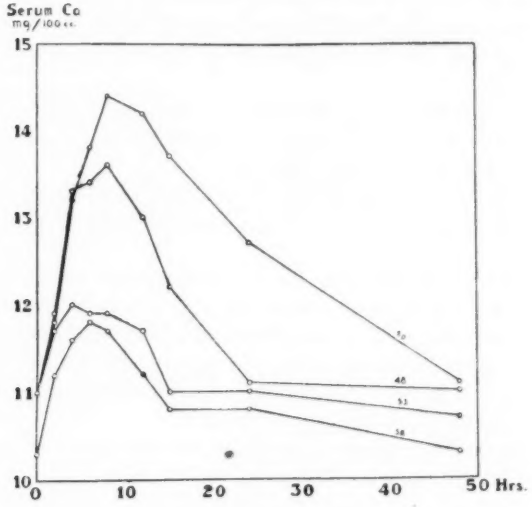

Fig. 3

injections. Further, the response obtained from intravenous injection of the hormone was considerably less. The curves obtained for dogs 48, 50, 53 and 56 following the intravenous injection of fifty units "Parathormone" are shown in figure 3. The same dogs given fifty units intramuscularly raised the serum calcium 3.6, 5.8, 2.2 and 3.1 mgm. respectively in sixteen hours.

SUMMARY

1. Cats have been found to show little or no rise in serum calcium within the first two hours after injecting parathyroid hormone intravenously, even when as much as one hundred units were given, and hence cannot be used,

as suggested by Stewart and Percival, in assaying extracts of the parathyroid hormone.

2. The potency of the active principle obtained by acid hydrolysis was not altered by making the hydrolysate alkaline to phenolphthalein.

3. Acid hydrolysis freed the active principle of the parathyroid glands, but too long hydrolysis destroyed it. Using 1.5, 3 and 5 per cent hydrochloric acid, the rate of destruction increased with the strength of acid used. With these strengths of hydrochloric acid, the most potent extract was obtained by hydrolyzing for forty-five minutes with 1.5 per cent acid.

4. The parathyroid glands exhibited a marked buffering action.

5. In the course of preparing active extracts, heating at 80° to 100° was found necessary in order both to free the active principle and to destroy enzymes which would otherwise inactivate the hormone.

6. Acid was also found essential for the freeing of the active principle from the glands.

7. Saponin, applied as in Harington's method for purifying insulin, did not separate active and inactive fractions from "Parathormone," but destroyed all activity originally present.

8. Gradually approaching the isoelectric point from the acid side yielded an amorphous precipitate without a separation into active and inactive fractions.

9. Saturation of an alkaline solution with carbon dioxide removed all the activity in an amorphous precipitate which came down about pH 6.8. Smaller amounts of carbon dioxide failed to yield an inactive fraction.

10. The crystals, obtained when the active carbon dioxide precipitate was dissolved in alkali and alcohol added, were shown to be inactive and later were identified as sodium bicarbonate.

11. Continuous extraction with absolute ethyl alcohol did not alter the composition of the isoelectric precipitate.

12. Continuous extraction, first with absolute normal butyl alcohol, and then with saturated aqueous butyl alcohol, did not alter the composition of the isoelectric precipitate.

13. Addition of potassium acid phthalate to a solution of the active principle did not remove an inactive fraction.

14. Dogs responded better and more uniformly on a meat diet than they did on an oatmeal diet. This uniformity was further improved by the addition of cod-liver oil. Increasing the readily available calcium by feeding calcium lactate did not bring about a more uniform response.

15. Extracts prepared from oatmeal gave no evidence for the presence in the cereal of an interfering or antagonistic factor to the parathyroid hormone.

16. Decrease in response with repeated injections was found to be due to an increased tolerance rather than a depletion of readily available calcium.

17. Intravenous injection of the parathyroid hormone gave the maximum elevation of the serum calcium in four to eight hours and the response was considerably less than when the hormone was given either subcutaneously or intramuscularly.

The writer wishes to express his sincere appreciation to Dr. J. B. Collip for his interest and suggestions during the course of this investigation.

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THE RECIPROCAL INNERVATION IN REFLEX CHANGES OF HEART RATE

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Received for publication July 2, 1931

The data reported in this paper were obtained during an investigation of reflex changes in blood pressure (Freeman and Rosenblueth, 1931). The heart rates were taken then in order to consider their influence on the blood pressure. We shall have to refer frequently to that publication, with which this one is intimately related.

The mechanism by which reflex changes in heart rate occur has not yet been definitely established. While some facts are classical and unquestioned, other points remain still a subject of controversy.

Since Cyon and Ludwig's (1866) investigation the increase of the inhibitory action of the vagus on the rate of heart when the depressor nerve is stimulated, has been abundantly confirmed.

Reflex inhibition of vagus tone is also well established (Knoll, 1881; MacWilliam, 1893; Hunt, 1899; Bainbridge, 1914).

The part played by the accelerators in reflex changes of heart rate has been diversely interpreted. A complete review of the literature on this subject would be out of place here.

Hunt (loc. cit.), who criticized earlier work, came to the conclusion that there is no reflex increase of accelerator tone. This view was later held by many other investigators. Recently, however, Cannon and Lewis (1927) have proved that, in unanesthetized animals with the vagi cut, the heart rate increases when they pass from rest to activity, and have stressed the importance of the anesthetics that had been used (ether, chloroform, etc.) as the cause of the "physiological maximum rate of the heart" (Tulgan, 1924, a and b).

Reflex decrease of accelerator tone has likewise suffered from contradictory testimony. It was denied or not considered at all by early authors. Bainbridge (loc. cit.) states that "in no case does stimulation of the central end of the vagus slow the heart by decreasing the accelerator tone." Brücke (1917 a, b), working without anesthesia, produced, after sectioning the vagi, a reflex fall in heart rate; the slowing disappeared when the inferior cervical sympathetic ganglia and the stellates were extirpated.

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The notion that there is a reciprocal relation of opposed impulses in cardiac innervation appears in some recent papers (Kisch and Sakai, 1923; Anrep and Segall, 1926), but we do not know of any work that deals with all aspects of the problem.

METHOD. The method has been described in detail in our paper on reflex changes of blood pressure (Freeman and Rosenblueth, loc. cit.). It will be briefly summarized here.

Cats were used; some had been sympathectomized previously, others were normal. In two cases acute extirpation of the stellate ganglia and the upper thoracic sympathetic chains was performed during the experiment. Dial "Ciba" was injected intraperitoneally (in doses between 0.5 and 0.7 cc. per kgm. body weight) as an anesthetic. By means of a mercury manometer changes in the blood pressure and heart rate were recorded. The changes in heart rate were investigated on stimulating centrally the cut left vagus and depressor, the sciatic, saphenous and median nerves, before and after severance of the right vagus, ligation of the adrenals or injection of curare. The stimuli used were weak (secondary coil set at 10 to 12 cm.) or strong (4 to 6 cm.) tetanizing currents or single make and break induction shocks at a rate of 4 per second.

RESULTS. Sixteen animals were used, seven sympathectomized and nine with the sympathetic intact. The results to be described were obtained usually several times (as many as 4) in each cat. The same responses persisted after the adrenals were ligated and curare injected.

A decrease of the heart rate was obtained on stimulation of the central end of the left vagus and depressor both in sympathectomized animals with the right vagus intact (see fig. 1 A) and in vagotomized animals with the sympathetic intact (see fig. 1 B).

An increase of the heart rate was obtained on strong tetanizing stimulation of the central end of the sciatic or median both in sympathectomized animals with the right vagus intact (see fig. 2 A) and in vagotomized animals with the sympathetic intact (see fig. 2 B).

In every case the results were consistent and the heart recovered afterwards its original rate.

Stimulation of the central end of the saphenous nerve with weak single induction shocks (4 per second), which elicited almost invariably a fall of blood pressure, did not usually produce any change of the heart rate. In two cases, however, in sympathectomized animals, we did observe a slowing of the heart.

In sympathectomized animals, with the vagi intact, struggle or movement produces an increase of the rate of the heart (see fig. 1 A, Freeman and Rosenblueth, 1931).

DISCUSSION. The changes of heart rate which occurred in our experiments can only be due to changes in the tone of the vagal or the accelerator system of the heart. All other factors were excluded.

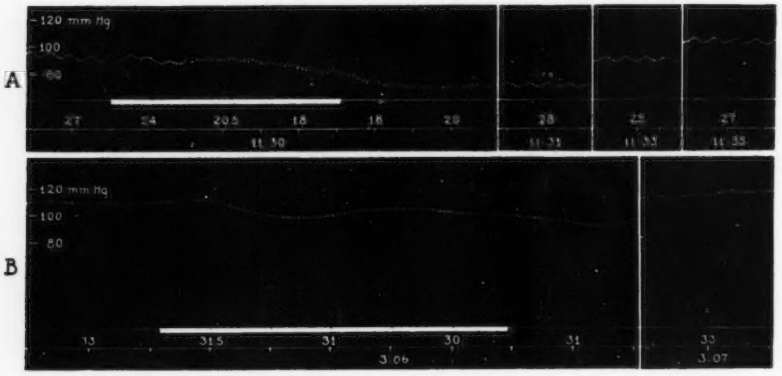

Fig. 1. Changes of heart rate on central stimulation of left vagus and depressor for 30 seconds. Dial, 0.7 cc. per kgm., intraperitoneally. Adrenals ligated. Curare. Artificial respiration.

A. Right vagus intact. Stellate ganglia and superior thoracic sympathetic chains extirpated.

B. Vagi cut in the neck; sympathetic intact. In this and other records the time is marked in five-second intervals and the figures indicate the number of heart beats per 10 seconds.

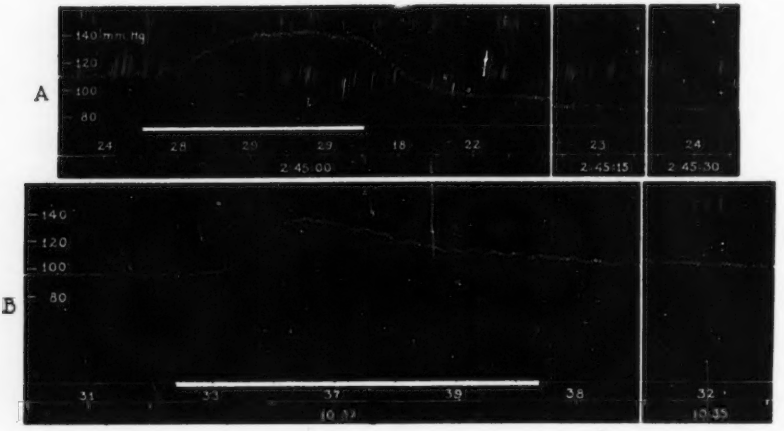

Fig. 2. Changes of heat rate on strong central stimulation of sciatic. Dial, 0.7 cc. per kgm., intraperitoneally. Adrenals ligated. Curare. Artificial respiration.

A. Vagi intact. Stellate ganglia and superior thoracic sympathetic chains extirpated.

B. Vagi cut in the neck; sympathetic intact.

The influence of adrenin was excluded by ligation of the adrenals.

The possible influence of respiratory changes (Hunt, 1899) was eliminated by curare and by artificial respiration.

Changes in blood pressure, within wide limits, do not directly affect the heart rate. Changes of intracranial pressure and pressure effects from the carotid sinus were not involved since in our experiments the arterial tension and the heart rate varied in the same direction, whereas the pressure changes mentioned produce opposite modifications of heart rate. It will also be shown later that the variations of the heart rate and of the blood pressure may be independent.

Alterations of heart rate due to changes of temperature could be produced only by a sudden flush of blood from the warmer abdomen when there is a vaso-constriction in that territory. Cannon and Rapport (1921) have shown that this cannot be an effective factor under the circumstances.

Since the changes of heart rate are, therefore, produced by modifications of the tonus of the inhibitor and the accelerator systems, and since we obtain both increases and decreases under identical conditions when either the vagi or the accelerators alone are active, we conclude that the sympathetic and parasympathetic systems are reciprocally related in reflex changes of heart rate.

The reflex changes in heart rate occur usually in the same sense as do those of blood pressure; that is, acceleration of the heart is accompanied by increase in blood pressure (see fig. 2), slowing of the heart is usually attended by a fall in blood pressure (see fig. 1). The detailed study of the tracings shows, however, that the two reflexes are independent.

At a given moment the heart rate may be increasing and the blood pressure falling, or *vice versa* (see all figures). The blood-pressure effect recovers usually before the change of heart rate has completely disappeared (see figs. 1 A and 2 A). In some cases only one of the responses may occur; this usually happens when sensory nerves are stimulated with weak, slow, single induction shocks. The blood pressure falls while the rate of the heart does not vary.

The increase of heart rate on struggle in sympathectomized animals can be due only to an inhibition of vagal tonus. As already mentioned, Cannon and Lewis (1927) proved that a similar increase, due to a stimulation of the accelerators, occurs in vagotomized animals with adrenals and liver denervated. We conclude, therefore, that here again there appears a reciprocal innervation of the heart.

SUMMARY AND CONCLUSIONS

The reflex changes of heart rate on afferent stimulation of different nerves (left vagus and depressor, sciatic, saphenous, median) were studied in cats under dial anesthesia, under curare and with the adrenals inacti-

vated, *a*, with vagi cut and sympathetic system intact; *b*, sympathectomized and with right vagus intact.

In both cases the same stimuli produced similar reflex increases and decreases of heart rate (see figs. 1 and 2).

It is concluded that reflex changes of heart rate involve a mechanism of reciprocal innervation.

The increase of heart rate accompanying struggle is shown to illustrate reciprocal innervation.

Evidence is adduced which indicates that reflex changes of heart rate may be independent of reflex changes in blood pressure.

We wish to express our appreciation and thanks to Prof. W. B. Cannon for his constant guidance and help which have made our year's stay in his laboratory a most pleasant and valuable experience.

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AN UNSUCCESSFUL ATTEMPT TO DEMONSTRATE HUMORAL ACTION OF "VAGUS SUBSTANCE" IN THE CIRCULATING BLOOD

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Received for publication July 2, 1931

Newton, Zwemer and Cannon (1931) and Cannon and Bacq (1931) found that a humoral substance, sympathin, is liberated into the blood stream when the sympathetic division of the autonomic nervous system is stimulated and that it has an adrenin-like action on distant organs which are subject to sympathetic control. A parasympathico-mimetic substance likewise has been argued for the cranial division of the antonomic system. The reasoning behind this view postulates that when the fibres of the cranial division are stimulated artificially this material is liberated and thus effects the local response. Loewi (1921) was the first to indicate such a substance. He found a relative bradycardia in a frog heart perfused by Ringer's solution taken from another heart after repeated vagus stimulation. He concluded that this substance was directly responsible for the slowing in both hearts. Brinkman and Van Dam (1922), extending Loewi's experiments, demonstrated a vago-mimetic action of the heart perfusate on the stomach of the second frog. Zunz and La Barre (1927) perfused an isolated cat's heart with Locke's solution during stimulation of the vagi; when the perfusate was injected into another animal a prompt slowing of the heart occurred, with a later return to the former rate.

Brinkman and v.d. Velde (1925) claim to have demonstrated the presence of this vago-mimetic substance in the blood of intact mammals. In one series of experiments (1925a) they injected carotid blood, obtained from an unanesthetized rabbit during direct and reflex stimulation of the vagus, into the ear vein of a second rabbit, and reported a slowing of the heart rate in the latter. In another similar experiment (1925b) they inferred an increase of tone of the stomach, associated with a diminution of peristalsis, in the second animal. Their methods and records are not convincing. Tournade, Chabrol and Malmejac (1926), using the heart as an indicator in crossed circulation experiments, were unable to confirm their results. Plattner (1926) likewise was unable to confirm the results of Brinkman and v.d. Velde, even though he drew the blood from the coronary

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sinus during vagus stimulation and injected it directly into the left auricle of the second animal.

The foregoing observations left open the question of the existence of a parasympathetico-mimetic substance carried in the circulating blood. Yet the testimony to its presence in Locke's solution, which had been in contact with organs under vagal influence, was fairly consistent. Might not the failure to demonstrate it in circulating blood be due to the use of too small an area of vagus distribution? If the whole vagal supply to the gastro-intestinal tract were employed as a source of the vagus substance perhaps enough would be produced to have effects. In that case the portal circulation might take on a new significance—it might protect the body from disturbing effects of a substance which, acting like acetylcholine, could lower blood pressure, for Hunt (1918a) showed that acetylcholine loses much of its depressive action on passing through the liver. Furthermore, sensitization might make possible the demonstration of vagus substance. Thus, structures might be sensitized to it, as Cannon and Bacq (1931) found that structures were sensitized to sympathin, by previous denervation. Also, there was the chance that just as organs can be sensitized to sympathin by cocaine (Rosenblueth and Schlossberg, 1931), so likewise they could be sensitized to vagus substance by physostigmine (Hunt, 1918b). It seemed worth while, therefore, to try to demonstrate the presence of vagus substance in circulating blood by excluding the liver and by applying methods of sensitizing responsive organs.

METHODS. Our experiments were performed upon varied types of preparations. Cats were used throughout. Anesthetics included ether, ether-curare, ether-decortication, ether-decerebration (Pollock and Davis, 1924), chloralose, chloralose-urethane and "dial" Ciba. In every case the anesthesia was as light as could be maintained and still prevent movement. In addition several preparations were not observed until they had recovered from the ether depression. Attempts to detect parasympathetic activity were made on the rate of the denervated heart, on blood pressure, on movements of the iris, on movement of skeletal muscles, on salivary secretion and on gastro-intestinal tonus and peristalsis. The heart rate was registered by use of either a mercury manometer or a Marey tambour placed against the chest. Blood pressure was recorded by the former method. Movements of the iris and the tongue were observed directly. Wharton's duct was cannulated and the salivary flow was recorded by a double-tambour recorder. Gastro-intestinal tonus and peristalsis were recorded by use of a balloon and tambour.

Sensitization of the structures was accomplished by aseptic denervation 5 to 10 days before the acute experiment. The ciliary ganglion and the superior cervical ganglion were excised to denervate the iris. The tongue was unilaterally denervated by section of the hypoglossal nerve. The sub-

maxillary gland was denervated by cutting the chorda tympani branch of the lingual nerve, excising the superior cervical ganglion and painting the hilus of the gland with phenol followed by alcohol, during pilocarpine secretion.

As cited above, Hunt (1918a) found that a dose of the parasympathicomimetic drug, acetylcholine, which would effect a marked fall of blood pressure when injected into a systemic vein, would have no effect on the blood pressure when injected into a mesenteric vein. Hence in the majority of our experiments we have shunted the portal circulation into the inferior vena cava by the method described by Firor and Eadie (1930). In addition, in certain experiments, the abdominal aorta just above the renal arteries and the inferior vena cava just above the renal veins, were ligated. Since Hunt (1918b) had found that physostigmine enhanced the action of acetylcholine, it was used in several experiments in an effort to obtain a response to vagus stimulation.

In the experiments on the gastro-intestinal tract the vagus trunk was cut low in the thorax and the entire esophagus severed. In the remainder of the experiments the peripheral vagus was stimulated in the neck or low in the thorax. In some instances the cardiac and pulmonic branches were cut, and in others they were left intact.

RESULTS. Our results were negative throughout. We used forty-three animals under different anesthetics or decerebration, as mentioned above. We excluded possible destructive action by the liver by connecting the portal vein to the inferior vena cava. We sensitized the test organs by denervation and possibly also by injection of physostigmine. Yet we were unable at any time to demonstrate the presence of a vagus substance or a substance whose action resembled that obtained on stimulation of the cranial parasympathetic nerves. In two experiments only did a slight slowing of the denervated heart occur during vagal stimulation of the gastro-intestinal tract. In these two cases ether was used as the anesthetic and a portal shunt had been performed; aseptic denervation of the heart antedated the acute experiment 8 days. The left peripheral vagus in the neck was brought through the incision and stimulation was delayed until the ether depression was absent. We were, however, unable to repeat these observations; indeed, we met repeated failures even after the injection of physostigmine. Since the rate of the denervated heart of these animals was over 200 beats per minute, and the animals were moving at intervals, it is probable that the observed decline in rate was due to a lessened secretion of adrenin. The negative results encountered in observations on the denervated iris seemed most definite. Stimulation of both vagi—innervating the heart, lungs and gastro-intestinal tract—in an animal with a portal shunt and injected with physostigmine, was followed by no change in the size of the iris, which had been completely denervated several days

previously. In this case the peripheral ends of the cut vagi were brought through an incision in the neck and were stimulated only after the ether narcosis had largely disappeared. The tongue gave an excellent example of the Vulpian-Philippeaux pseudo-muscular contraction (1863) on stimulation of the lingual nerve and on injection of acetylcholine, but failed to respond on stimulation of the vagi going to the heart, lungs and intestines.

A protocol showing the procedures in a typical experiment is given below:

January 16, 1931

10:00 a.m. Ether started. Tracheal insufflation. Mid-line incision from 1 cm. below symphysis of mandible down to point just above superior border of sternum. Right chorda tympani branch cut as it leaves the lingual nerve. Right hypoglossal nerve cut above ansa hypoglossa. Right superior cervical ganglion isolated and excised.

10:15 a.m. One-tenth cubic centimeter of a 1 per cent pilocarpine solution injected into sternohyoid muscle. Right Wharton's duct dissected free about hilus of right submaxillary gland and painted with phenol. Seventy per cent alcohol applied to same area 10 seconds later.

10:30 a.m. Layer closure without drainage.

January 18, 1931.

Dressing off. Wound not infected. Cat in excellent condition but has some difficulty in eating because of unilateral (right) denervation of tongue.

January 27, 1931.

1:55 p.m. Six and seventy-five hundreths cubic centimeters 2 per cent solution of chloralose intraperitoneally.

3:00 p.m. Tracheal cannula. Both vagi cut in neck and separated from sympathetic trunk. Cannula in left carotid artery connected with mercury manometer.

3:30 p.m. One-tenth cubic centimeter 1 per cent solution of pilocarpine intramuscularly.

3:40 p.m. Salivary cannula in right submaxillary duct and arranged to record drops of saliva (Cannon and Bacq, 1931).

4:20 p.m. One-tenth cubic centimeter 1 per cent pilocarpine solution injected intramuscularly.

4:30 p.m. Effect of pilocarpine subsiding. Both vagi, peripheral in neck, stimulated by Harvard inductorium 30 seconds. Secondary at 8.0 cm. No change in rate of salivary flow.

5:00 p.m. Animal moving. One cubic centimeter chloralose intravenously.

5:10 p.m. Both vagi stimulated 30 seconds. Secondary coil at 8.0 cm.

5:20 p.m. One-tenth cubic centimeter 1 per cent pilocarpine solution injected intramuscularly.

5:30 p.m. Effect of pilocarpine subsiding. Stimulation repeated, 60 seconds. No change in rate of salivary flow.

5:40 p.m. Five-tenths cubic centimeter 1:100,000 acetylcholine in 5 seconds. Marked increase in salivary flow.

5:50 p.m. Stimulation repeated 60 seconds. No effect on movement of tongue.
6:00 p.m. One cubic centimeter 1:100,000 acetylcholine. Marked contraction of denervated tongue.
6:10 p.m. Stimulation repeated, 60 seconds. No effect on tongue.

Since our results were so consistently negative, we are not publishing records, charts, graphs, etc., of our experiments.

DISCUSSION. That we were unable to demonstrate the presence of a vagus or a parasympathico-mimetic substance in the intact animal accords with the results of Tournade, Chabrol and Malmejac (1926) and Plattner (1926). Both Plattner (1926) and Zunz and La Barre (1927) showed how readily the vagus substance is destroyed on subjecting it to the action of blood. Again Matthes (1930) showed that acetylcholine, a parasympathico-mimetic drug, is destroyed by an esterase in the circulating blood, a suggestion first made by Dale (1914). In all experiments in which the hypothetical substance passes from one organ to another it is necessarily in contact with blood for some time. We shortened the period of contact with the blood (and the contact with the liver) by means of a portal shunt, and we used physostigmine in an effort to inhibit the esterase action and yet could not demonstrate the vagus substance. With the exception of Brinkman and v.d. Velde (1925a and b) all investigators who have tried to demonstrate the presence of this substance in the blood of mammals during vagal stimulation, have failed. And although Plattner (1926) and Zunz and La Barre (1927) found it in the Locke solution perfusate in mammalian experiments, they were unable to demonstrate in the blood of the animals they studied.

CONCLUSION

In an effort to demonstrate the presence of vagus substance in circulating blood, we experimented on 43 cats under different anesthetics or decerebrated. In order to obtain as much vagus substance as possible, the entire vagal supply to the smooth muscle of the alimentary canal was used as a source, and in some experiments the supply to the heart and lungs was added or used alone. The portal vein was connected to the inferior cava in order to avoid possible destructive action on the vagus substance by the liver. A variety of test organs—iris, submaxillary gland, heart, tongue, stomach and intestines—was employed, some of them sensitized by previous denervation. Physostigmine was given also as a means of perhaps enhancing the action of vagus substance. Our results were wholly negative.

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THE INFLUENCE OF RATE OF SECRETION ON THE CONCENTRATIONS OF POTASSIUM AND SODIUM IN DOG'S SUBMAXILLARY SALIVA

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Received for publication June 10, 1931

Considerable confusion exists in modern physiological, biochemical and dental literature regarding the concentrations of inorganic constituents in saliva, mainly because investigators concerned with this problem have failed to take into account the importance of the secretion rate as a factor determining the composition of saliva. Long ago Heidenhain (1878) showed that the total percentage of inorganic salts in saliva becomes greater as the rate of secretion is increased (Heidenhain's law). His observations were confirmed by Werther (1886), by Langley and Fletcher (1889), and by Zebrowski (1905). In spite of this accumulated evidence, it has received little attention. A number of investigators have studied the changes in hydrogen-ion concentration and composition of saliva in response to changes in diet, etc., without sufficient regard for the changes in composition that may be brought about by mere alteration of the rate of secretion. This has led to inconsistent results, as may be seen from the following quotation,—"Having been unable to show any constancy in the composition of saliva under regulated dietary conditions, and finding as great variation under fasting conditions as when food was ingested, it seemed inadvisable to attempt further studies with diets as we had originally planned to do" (Clark and Levine, 1927). As will be seen later, however, when concentrations are arranged with reference to the rate of secretion they do bear some semblance of order.

Sodium and potassium are the most abundant basic constituents in saliva. This is shown in table 1. The first two columns give some figures from analyses of the ash of human saliva; the figures in the third column are averages of values obtained by Clark and Shell (1927) in their extensive study of "resting" saliva from normal men; in the last two columns are shown the concentrations of the four basic cofistituents in human blood plasma, and comparison of these with the figures for human saliva shows the extraordinary selective action of the secretory cells.

The purpose of the present investigation was to determine the effect of changes in rate of secretion upon the concentrations of potassium and

sodium in saliva. A similar type of experiment was attempted by Werther in 1886. He determined the percentage of water-soluble and of water-insoluble salts in the ash of dog's chorda saliva obtained at different rates of secretion. From the description of his chemical procedure it follows that the "unlösliche Salze" were largely, if not entirely, calcium salts. Figure 1 gives the results of one of his experiments.

METHODS. Of the four experiments reported in this paper, two (fig. 2, A and B) deal with pilocarpine saliva. The results presented in figure 2A were obtained on saliva collected from a dog with a permanent submaxillary fistula. A flow of saliva was induced by intravenous injection of a 0.01 per cent pilocarpine solution, the rate of injection being regulated by a Colwell pump (Colwell, 1930). Aseptic precautions were taken and no anesthesia was required. To avoid, so far as possible, the effect of previous pilocarpine stimulation of the gland, only one or two samples were

TABLE 1

	(HAMMERBACHER, 1881) IN 100 GRAMS ASH	(JACOBI, 1896) IN 100 GRAMS ASH	(CLARK AND SHELL, 1927) PER 100 CC. OF "RESTING" SALIVA		(PETERS AND VAN SLYKE, 1931) PER 100 CC. OF HUMAN PLASMA	
	grams	*grams*	*mgm.*	*cc. 0.1 N*	*mgm.*	*cc. 0.1 N*
Cl	18.35	14.07	50	14.1		
Na	9.59	26.96	20.8	9.0	335	146
K	45.71	35.78	55.4	14.2	19.5	5.0
Ca	5.01	3.07	7.2	3.6	9.6	4.8
Mg	0.16	0.55	0.7	0.6	3.2	2.6

collected each day, until samples had been obtained at widely different secretion rates.

The results in figure 2B (expt. 5) were obtained on pilocarpine saliva from a dog anesthetized with "dial" (Ciba). All branches of the left carotid artery above the lingual, except the one supplying the submaxillary gland, were tied. The lingual was then cannulated with a long piece of flexible metal tubing connected to a so-called "intra-arterial pump." This pump consisted of a 10-cc. syringe, the plunger of which was moved forward slowly by a rack driven through a set of 17,000:1 reduction gears by an electric motor. The syringe delivered from 2.5 to 11 cc. per hour, depending upon the resistance placed in series with the motor. With this method an abundant flow of saliva could be called forth from the injected gland without introducing a sufficient amount of pilocarpine into the body to produce the usual circulatory disturbances, nor even to call forth a secretion from the other salivary glands. Wharton's duct was cannulated and connected with a 5-cc. burette graduated in 0.01 cc. The rate of secretion was measured from time to time with the aid of a stop watch. In experi-

ment 5, 0.02 per cent pilocarpine in 0.9 per cent saline was injected into the lingual artery at the rate of 11 cc. per hour for nearly two hours, during which time the salivary flow fell from nearly 60 to 4 cc. per hour. The saliva was divided into four samples for each of which the average secretion rate was known.

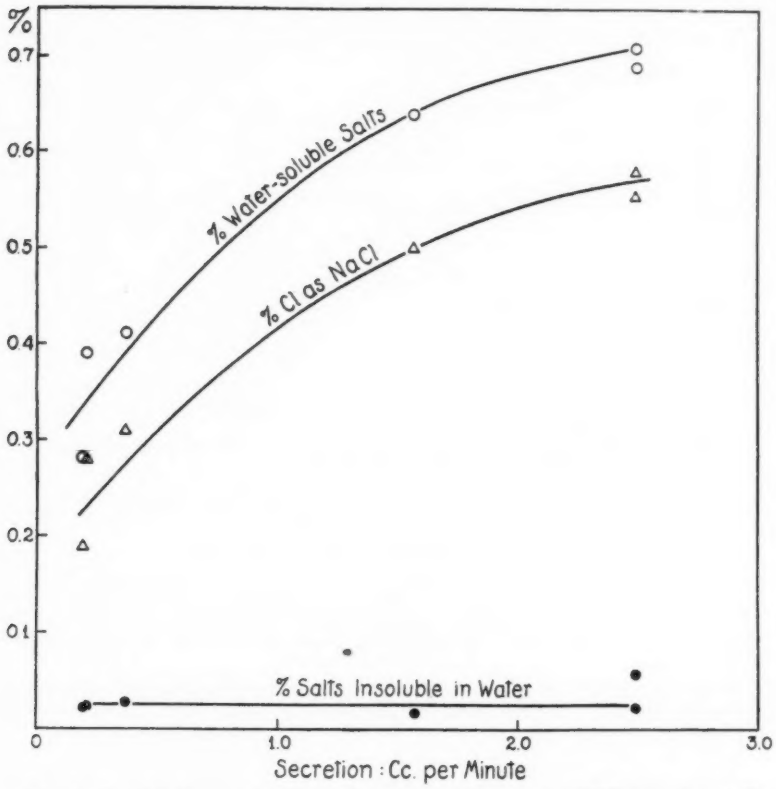

Fig. 1. Graphical presentation of the results of Werther's (1886) experiment III showing the relation between the rate of secretion and percentage of inorganic salts in dog's chorda saliva.

The other two experiments reported here (expts. 7 and 8, see fig. 3) deal with dog's chorda saliva. The dogs were anesthetized with "dial." Shielded electrodes were placed on the severed chordo-lingual nerve and connected to the secondary of an inductorium. The primary coil was connected in series with one dry cell, a simple key and a circuit breaker

operating at about 40 per second. Fifteen seconds of stimulation were alternated with fifteen seconds of rest until the necessary 4 to 5 cc. of saliva had been collected. The strength of stimulus was varied by changing the position of the secondary coil. The secretion rate was measured as in experiment 5.

Sodium was determined by the method of Barber and Kolthoff (1928),

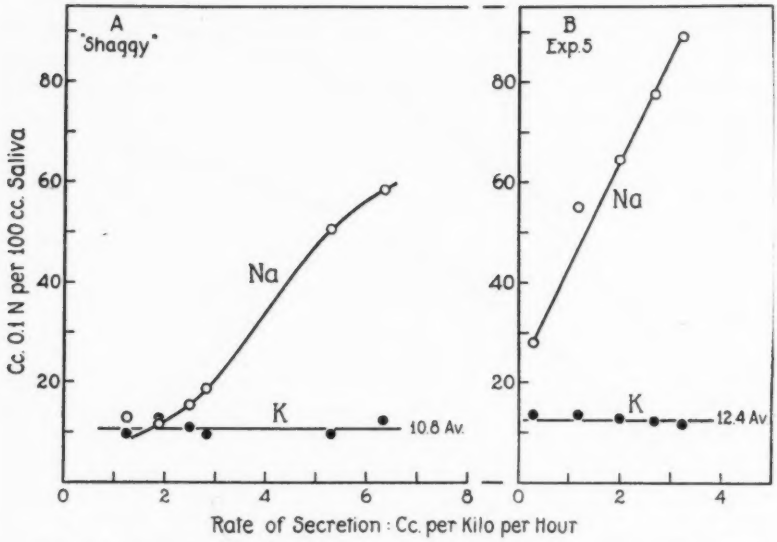

Fig. 2. A. "Shaggy," male dog 17 kgm. with permanent submaxillary fistula. Dilute pilocarpine solution (0.01 per cent) injected intravenously. The graph shows potassium and sodium concentrations in samples of submaxillary saliva secreted at different rates.

B. Concentrations of sodium and potassium in the saliva, at the average secretion rates, in a dog (17 kgm.) receiving 0.02 per cent pilocarpine in the artery of the left submaxillary gland at the rate of 11 cc. per hour for nearly two hours.

and potassium by Fiske's method (personal communication). No phosphate was found in any of the samples analyzed.

Results and discussion. The results are presented in the form of graphs (figs. 2 and 3) in which the ordinates represent concentrations of sodium and potassium in cubic centimeters 0.1 N per 100 cc. of saliva. The abscissae represent rates of secretion as cubic centimeters per kilogram body weight per hour. Since the weight of the submaxillary gland is roughly proportional to the body weight, this gives the rate in terms comparable to cubic centimeters per gram of gland per hour, and therefore permits a comparison of the results obtained on different dogs (see fig. 3).

A glance at figures 2 and 3 will show that the sodium concentration in saliva is subject to wide variation, depending upon the rate of secretion, whereas the concentration of potassium is practically constant and independent of the rate. At rapid rates of secretion sodium is more abundant than potassium but at slow rates it falls below potassium in concentration.

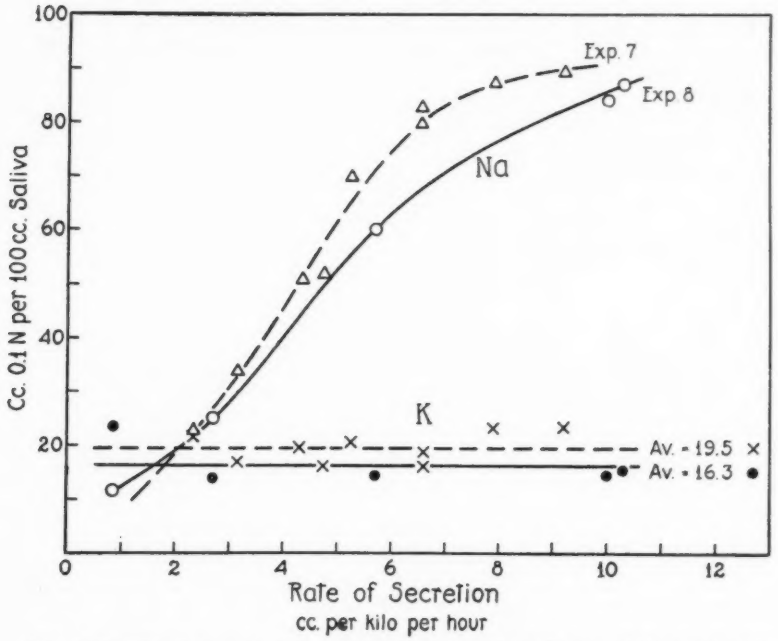

Fig. 3. Graphical presentation of results, showing the influence of changes in rate of secretion upon the concentration of sodium and potassium in dog's chorda saliva.

SUMMARY

In dog's submaxillary saliva (resulting from chorda or pilocarpine stimulation) the sodium concentration varies between 10 and 90 cc. of 0.1 N per 100 cc. of saliva, dependent closely on the rate of secretion. The potassium concentration, on the other hand, does not vary with the secretion rate and remains practically constant at a level of 10 to 20 cc. of 0.1 N per 100 cc. of saliva.

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STUDIES ON THE CONDITIONS OF ACTIVITY IN ENDOCRINE ORGANS

XXVII. Evidence that Medulliadrenal Secretion is not Continuous

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Received for publication June 16, 1931

One of the early theories of the function of secreted adrenin was that it exercises a continuous tonic influence on structures under sympathetic control. This theory involved the assumption that there is a continuous secretion into the blood stream. Support for the view was found in the experiments of Strehl and Weiss (1901) who reported a fall of blood pressure in rabbits immediately after closure of the adrenal vessels and a prompt rise when the vessels were opened. Kahn (1911), however, was unable to confirm these experiments—when he tied the adrenal vessels in rabbits the pressure did not fall. And Hoskins and McClure (1912) found that the procedure had no effect on the blood pressure of dogs. Other investigators, including Lewandowsky (1899), Camus and Langlois (1900) and Trendelenburg (1914) have made observations in accord with these. The evidence at hand, therefore, appears to weigh heavily against the idea that the vascular tone of blood vessels depends on a sustained discharge of adrenin.

Elliott (1904) suggested that minute amounts of circulating adrenin are necessary for the efficacy of sympathetic impulses. By test on a series of dogs, normal or deprived of their adrenal glands, that were given slow infusions of dilute adrenalin, Hoskins and Rowley (1915) were unable to demonstrate that the greater concentration of adrenin in the blood ever had the facilitating influence that had been postulated.

The observation of Aub, Bright and Forman (1922) that adrenalectomy lowers the basal metabolic rate and that intravenous infusion of adrenalin restores it has been used to support the argument for a tonic service of medulliadrenal secretion (cf. Bayer, 1929, p. 481). The argument is not compelling, however, for the reduced metabolism after adrenalectomy may be due to removal of the cortical tissue; the ability of adrenalin to raise the metabolism, under the circumstances, may be quite as independent of the adrenal cortex as it is independent of the thyroid (cf. Aub, Bright and

Forman 1922); and this ability is no more proof that adrenalectomy deprives the body of the benefits of continuous discharge of adrenin than that thyroidectomy has that effect.

The distinction which should be drawn between effects of total extirpation of the adrenals (medulla and cortex) and extirpation of the *medulla alone* is emphasized by observations on blood sugar in these two conditions. Bornstein and Holm (1922) and Banting and Gairns (1926) found that adrenalectomy caused hypoglycemia, but Frank and Isaac (1910), Kahn and Starkenstein (1911), Stewart and Rogoff (1918) and others have shown that destruction of the medullary substance does not reduce the glycemic level. It appears that the percentage of blood sugar is not dependent on a continuous supply of adrenin to the blood.

The "tonus theory" of medulliadrenal function has been attacked by others who have examined the effectiveness of sympathetic nerve impulses either directly or reflexly aroused, before and after excluding participation of the adrenals. Gley and Quinquaud (1918, 1921) have been especially vigorous in this attack. They demonstrated that vagal and depressor stimulation, as well as accelerator, had the same influence on the heart rate, and that splanchnic stimulation and asphyxia had the same effect on the blood pressure, whether the adrenal glands were present or not.

A theory of medulliadrenal function alternative to the tonus theory is that adrenin is secreted in times of special stress, when it coöperates with sympathetic nerve impulses in rendering the defense or protection of the organism more effective (cf. Cannon, 1914, 1928). There is no logical antagonism between the "tonus" theory and the "emergency" theory of the service of adrenin—adrenin might ordinarily be secreted continuously at a slow rate and the continuous output might be augmented when the sympathico-adrenal apparatus is brought sharply into action, as in emotional excitement, pain, cold, fever, asphyxia and hypoglycemia. Although the two theories might thus both be correct, however, the lack of cogent proof of continuous secretion of adrenin has tended to relegate the tonus theory to the background.

Fairly recent observations have furnished evidence which might be interpreted as favorable to a normal, continuous discharge of adrenin and which has in fact led to the inference that adrenin has a tonic effect. Stewart and Rogoff (1919) have reported a uniform unvarying secretion, which reaches the heart in sufficient concentration to exert upon it a definite action. Cannon and Rapport (1921) observed that the denervated heart in animals under ether or urethane beats considerably more slowly after removal of the adrenal glands, without, however, noteworthy fall of blood pressure. Tournade and Chabrol (1922) found that after adrenalectomy the blood pressure of a dog (A), under chloralose anesthesia, fell progressively; when the right adrenal vein of another dog (B), likewise under

chloralose, was anastomosed with the jugular of A, the blood pressure of A promptly rose; when the right splanchnic of B (previously deprived of his left adrenal gland) was sectioned, the blood pressure of A fell from 160 to 100 mm. Hg; and when the peripheral part of the cut right splanchnic of B was stimulated the pressure of A rose again to 160. They drew the conclusions that the adrenal glands by internal secretion play an undeniable rôle in the maintenance of arterial pressure, and that secretion of adrenin is continuous, due to tonic nerve impulses, as proved by the fall of blood pressure in dog A after splanchnectomy in B. Heymans (1929) confirmed these "important facts"—the pressure of an adrenalectomized dog falls progressively, and after some hours is as low as 70–80 mm. Hg, whereas it soon rises if supplied with blood from the adrenal vein of another dog. These observations, he declares, seem to demonstrate in an indisputable manner that adrenalinemia plays a continuous and not an intermittent cardio- and angiotonic rôle.

In the foregoing brief review it is noteworthy that neither Stewart and Rogoff nor Cannon and Rapport regarded the evidence that adrenin, secreted in sufficient amount to have hormonal effects on the heart, has important tonic action. Indeed, one may grant such effects as they observed, and also the effects noted by Tournade and Chabrol and by Heymans, but may still question whether they indicate a *normal* relation of medulliadrenal secretion to the functioning of the circulatory system. It is quite possible, on the basis of the emergency theory, that the manipulation, surgical interference and the excitement of entering anesthesia, that attends the acute experiment, may stimulate medulliadrenal secretion —a secretion which is quite unnatural for undisturbed animals. Thus the average figure of fifteen sets of determinations by eight different investigators for the output of adrenin from the adrenal glands in the resting state is 0.00055 mgm. per kgm. per minute (cf. Bayer, 1929, p. 551). But that amount is 10 times the minimal amount capable of producing hyperglycemia in the rabbit and 20 times the minimal amount for man (cf. Cori, 1931, p. 216). One-half that amount causes glycosuria (Trendelenburg, 1923a). Since glycosuria is abnormal this output of adrenin must be abnormal. It is obvious that this so-called "resting state," therefore, accompanied by operative disturbance of the organism, causes a considerably exaggerated medulliadrenal secretion. Indeed, as Trendelenburg (1923b) remarked, the true secretory activity in the resting animal is quite unknown.

On abolishing, by adrenalectomy or cutting the secretory nerves, the extra secretion induced under experimental conditions, characteristic atonic effects might result because the spontaneously secreted adrenin has been exerting a tonic influence. But, with that granted, is it possible that some spontaneous secretion goes on under natural, calm conditions?

Cats with denervated hearts have been observed in this laboratory for many days before and after inactivation of the adrenal medulla (removal of one, and complete denervation of the other by severance of the splanchnics and extirpation of the upper abdominal sympathetic chain) and before and after removal of the medullary portion of the remaining adrenal. The denervated heart is exquisitely sensitive to variations in the adrenin content of the circulating blood—1 part adrenin in 1,400,000,000 parts of blood causes a faster pulse (Anrep and Daly, 1925). If there is a continuous secretion of adrenin, therefore, the rate of the denervated heart should be distinctly lower after inactivation of the adrenal medulla whether by denervation or destruction.

Actual observations show that the drop in heart rate, which would indicate removal of the influence of circulating adrenin after exclusion of medulliadrenal influences, does not occur. In table 1 are assembled data already published in the paper by Cannon and Britton (1927), showing the lowest basal rates of the denervated heart in seven different animals before and after medulliadrenal inactivation. The *lowest* rates were taken from the observations made before that operation because of the readiness with which slight disturbances of the animals would cause uneasiness, and consequent discharge of adrenin and a faster pulse. There was not that danger after the operation, but, as Moore (1929) proved, protein food can raise the pulse rate, and therefore the lowest rates were selected also from among those recorded when the output of adrenin was no longer under nervous control. Comparison of the lowest basal rates before and after suppression of the medulliadrenal output by severance of the secretory nerves reveals no clear evidence in favor of continuous secretion of adrenin, at least to a degree which would have physiological effects on an organ sensitized by being deprived of its immediate sympathetic nerve supply. In two of the seven animals, 23 and 46, the lowest basal rate before was higher than it was after the operation. This might readily be due to failure to wait sufficiently long to permit the animal to become quite serene. In the other five cases the rates before were about the same as after or were somewhat lower. This is a result not favored by the conditions of the experiments, for, with the adrenal glands present and normally innervated, an accelerating effect on the denervated heart is hard to avoid.

These observations are confirmed by results derived from animals the beats of whose denervated hearts were recorded before and after exclusion of adrenin effects by *destruction of the medulla* of the remaining adrenal gland. Reference to table 2 shows that the basal heart rates do not differ consistently and to a noteworthy degree after the operation as compared with before.

The similarity of the figures reported in tables 1 and 2 indicates that after severance of the secretory nerves (in the cases in table 1) no other agency

was causing a discharge of adrenin, i.e., that denervation is equivalent to removal. It indicates also that there is no continuous "paralytic secretion" of adrenin that follows denervation, as Tournade (1931) has inferred. The failure of Tourande to remove the upper abdominal sympathetic chains, as well as the splanchnics, would explain the persistence of some discharge of adrenin, for there are secretory fibres below the splanchnic trunks (cf. Stewart and Rogoff, 1917; and Cannon, Lewis and Britton, 1926).

The results obtained by records of the denervated heart are confirmed by observations on basal metabolism. McIver and Bright (1924) found that

TABLE 1

Lowest basal rates of the denervated heart before and after medulliadrenal inactivation by nerve section

CAT NUMBER	BEFORE (DATE)	DATE ADRENALS INACTIVATED	AFTER (DATE)
23	90 (February 5)	February 11	76 (February 12)
26	90 (March 22)	March 25	104 (April 1)
27	92 (April 6)	April 6	92 (April 13)
33	116 (April 10)	April 30	122 (May 5)
38	118 (June 1)	June 4	120 (June 5)
46	124 (June 21)	June 24	100 (June 28)
49	100 (June 21)	June 24	101 (June 28)

TABLE 2

Lowest basal rates of the denervated heart before and after medulliadrenal inactivation by removal of the adrenal medulla

CAT NUMBER	BEFORE (DATE)	DATE ADRENALS INACTIVATED	AFTER (DATE)
36	104 (February 28)	March 10	102 (March 17)
83	102 (January 22)	January 26	96 (January 31)
113	102 (March 3)	March 9	96 (March 11)
265	106 (March 5)	March 6	110 (March 16)

reflex or direct nervous stimulation of medulliadrenal secretion caused an increased metabolic rate, averaging about 20 per cent above the previous level. If there is a continuous discharge of adrenin under standard basal conditions, there should be a fall of the basal rate when secretory nerves are no longer acting. Cannon, Newton, Bright, Menkin and Moore (1929) found, however, no considerable alteration of the degree of metabolism of animals after sympathetic nerve impulses were completely excluded.

In previous publications (Cannon, 1915, 1930) emphasis has been laid on the arrangement of the sympathetic division of the autonomic system for diffuse discharge,—a concept supported by the diffuse distribution

of adrenin, acting as it does, synergistically with sympathetic impulses. In short, the sympathico-adrenal system would work as a whole. As Bard (1928) has pointed out, however, the integrating center for the sympathico-adrenal system is in the diencephalon, and this center plays on subsidiary centers which may be capable of more or less independent action. Thus the vasomotor center in the medulla retains its tonic activity after being disconnected from the mid-brain. From the data presented in the foregoing pages it would appear that the tonic activity of the vasomotor apparatus does not involve a tonic activity of the adrenal medulla. When, now, the integrating center in the diencephalon becomes dominant—as in emergencies, for example, or in the need of preserving homeostasis—there would be not only increased tone of the vasomotor center, but a reinforcement of that effect by adrenin specially discharged into the blood stream. As found by Cannon and Britton (1927), quite minor activity, such as walking, may be attended by a well marked output of adrenin.

SUMMARY

Observations for and against the continuous secretion of adrenin are reviewed, and the point is made that the evidence in favor of continuous secretion has been obtained under disturbing experimental conditions.

Records taken from quiet, healthy animals, with hearts denervated for some time and thereby especially sensitized to the action of adrenin, reveal no noteworthy differences between the basal heart rates before and after the adrenal medulla was inactivated, either by denervation or extirpation (see tables 1 and 2).

Since the heart, even when freshly denervated, is, to a very high degree, sensitive to circulating adrenin, the conclusion is drawn that adrenin is not secreted continuously, but, as other experiments have shown, only in emergencies or for the preservation of homeostasis.

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REFLEX STIMULATION AND INHIBITION OF VASODILATORS IN SYMPATHECTOMIZED ANIMALS

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Received for publication July 2, 1931

Cyon and Ludwig (1866), who discovered the depressor nerve, regarded its action as purely inhibitory on the vasoconstrictor center. This opinion was held by subsequent workers until the appearance of the paper by Bayliss in 1893. In this paper he gave evidence to show that the vasodilators were reflexly excited on stimulation of the depressor. Hunt (1895) likewise concluded from his studies on the action of afferent nerves on blood pressure that the fall in blood pressure accompanying certain types of stimulation of sensory and mixed nerves was due to an excitation of vasodilators. He believed, however, that stimulation of the depressor nerve inhibited vasoconstriction since the dilation took place mainly in the splanchnic area and was not affected by curare. Finally Bayliss (1908) brought forward the concept of the reciprocal innervation in vasomotor reflexes which he first suggested in 1893. According to this concept a pressor response, such as may be obtained on stimulation of the central end of the sciatic with a strong tetanizing current, is associated with a stimulation of the vasoconstrictor center and an inhibition of the vasodilator center. Conversely, a depressor response, following stimulation of the central end of the depressor nerve, is associated with an inhibition of vasoconstrictors and a stimulation of vasodilators.

Barcroft (1914), finding a rise in the metabolism of the submaxillary gland associated with vasodilation even when atropine had paralyzed secretion, believed that the vasodilation was caused by products of cell metabolism and even questioned the existence of vasodilator nerves. Gaskell (1916), summarizing the evidence for and against the existence of vasodilator fibres, concludes that the increased blood flow through a muscle or an organ is chiefly due to the direct action of acid metabolites from the small blood vessels, but at the same time admits the possibility of special vasodilator nerves. Tigerstedt (1923) after reviewing the entire literature states that the rôle of the vasodilator center in vasomotor reflexes is still open to question.

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The part played by the vasoconstrictors or the vasodilators in vasomotor reflexes can best be determined in the absence of one or the other system. The presence of vasodilators in the posterior roots of the entire cord, and in the anterior roots of the sacral cord and in some cranial nerves renders the extirpation of this system practically impossible. Cannon, Lewis and Britton (1927) have shown, however, that exclusion of all sympathetic nerve impulses is quite compatible with existence.

The only constrictor fibres of non-sympathetic origin actually known are those probably carried by the vagus to the coronary and pulmonary vessels (the latter have been shown only in cold-blood animals). Porter (1899), Maas (1899) and Wiggers (1909) found diminished flow through the coronary vessels on stimulation of the vagus. Porter (loc. cit.) observed a contraction of the coronaries even in the inactive heart. The same diminished flow occurs in the innervated heart-lung preparation when the vagi are stimulated (Anrep and Segall, 1926). These authors observed also a dilation of the coronaries when the vagi were cut. As stated above, the pulmonary vessels of cold-blooded animals are constricted by vagal stimulation (Couvreur, 1889; Cavazzani, 1891; Mashima, 1921; Luckhardt and Carlson, 1921). As will be shown later, however, the results which we shall report were obtained whether the vagi were cut or intact. The present paper is an account of vasomotor reflexes in the absence of known vasoconstrictor influences.

METHOD. Cats were used throughout. Sympathectomy was performed with slight modifications of the method described by Cannon, Newton, Bright, Menkin and Moore (1929). The thoraco-lumbar sympathetic chains were removed on each side from the stellate ganglion to the ganglion below the sacral prominence. No attempt was made to remove the cervical sympathetic, the semi-lunar or the superior and inferior mesenteric ganglia, since all connections of preganglionic fibres to these ganglia had been severed. The sympathectomized cats were used between 5 and 28 days after the final operation. In every case a post mortem examination was made to verify the completeness of the removal of the sympathetic chains. Normal animals were studied as controls.

Vasomotor reflexes produced by afferent stimulation of the depressor nerve or the depressor fibres contained in the left vagus, and of the sciatic or median and the saphenous nerves, were investigated. That afferent stimulation of the depressor or the depressor fibres in the vagus produces a fall in blood pressure, and that strong tetanizing afferent stimulation of the sciatic causes a rise, is well known. As regards the saphenous nerve evidence differs. Hunt (1895) was able to obtain only pressor responses from this nerve in the cat. Martin and Lacey (1914), however, evoked reflex depressor responses from the saphenous nerve in the cat by using threshold stimulation, thus proving that this nerve does not differ from

other sensory nerves in that regard. Gruber (1916) confirmed their findings.

Excitation of the depressor, sciatic or saphenous nerves was produced with a "Harvard" inductorium, 6 volts being used in the primary circuit. With the secondary coil set at 10 cm. the stimulus is barely perceptible on the tip of the tongue; at 8 cm. it tends to become painful. Gruber (1916) showed that afferent stimulation of sensory or mixed nerves by single induction shocks of weak intensity usually occasions a fall in blood pressure. Accordingly, when the saphenous nerve was stimulated to elicit a fall in blood pressure, a slowly interrupted current (4 makes and breaks per second) of weak intensity (coil distance, 10–12 cm.) was used. When the sciatic was stimulated to produce a rise in blood pressure or the depressor to produce a fall, tetanizing currents of moderately strong intensity (coil distance, 6–8 cm.) were used.

Dial, "Ciba," was the anesthetic. The drug was given intraperitoneally and occasionally intravenously in doses of 0.5 to 0.7 cc. per kgm. body weight. Ether was administered in two experiments but was found to mask the reactions. With dial both pressor and depressor responses were easily and consistently obtained.

Curare was used in order to rule out the effects of movement on the blood pressure. Gaskell (1876) found that if this drug abolishes muscular contraction it may, if the doses are large, also prevent the vascular dilation incident to stimulating the motor nerve to the muscle. The dose of curare used in our experiments was just sufficient to abolish visible movement—an amount which still permits vasodilation. It was given slowly by vein and artificial respiration administered.

As soon as the animal was anesthetized it was placed over a heating pad on the animal board. Cannulae were inserted into the trachea and into the right carotid artery. The blood pressure was recorded by a mercury manometer. The left vagus, separated from the sympathetic, was cut low in the neck, and the central end made ready for stimulation (the depressor, if separate, was incorporated with the vagus). A similar procedure was followed with the sciatic, or the median, and with the saphenous.

Records were taken before cutting the right vagus in the neck, after cutting it and after tying off both adrenals. The adrenals were ligated to prevent any possible changes in blood pressure due to variations in the secretion of adrenin. This operation was performed by the posterior approach in order to avoid disturbance of the peritoneal cavity. Adrenal ligation was not performed in the sympathectomized animals, since the glands were not connected with the central nervous system.

RESULTS. *Blood pressure reactions to struggle.* When animals which had been completely sympathectomized struggled for a short time, occasionally they lost consciousness, became limp and ceased breathing.

The heart beat was barely palpable. Soon after, gasping respirations started, and the animal recovered. In one cat the anterior spinal vessels were injured during extirpation of the abdominal sympathetic. When it recovered from anesthesia the hind limbs were paralyzed. This animal was used to determine the blood pressure reactions to struggle. A cannula was inserted into the femoral artery without anesthesia, since the hind limbs were insensitive, and by different stimuli the animal was caused to struggle. Figure 1 A shows the prompt fall in blood pressure associated with struggle. There was at the same time an *increase* in heart rate.

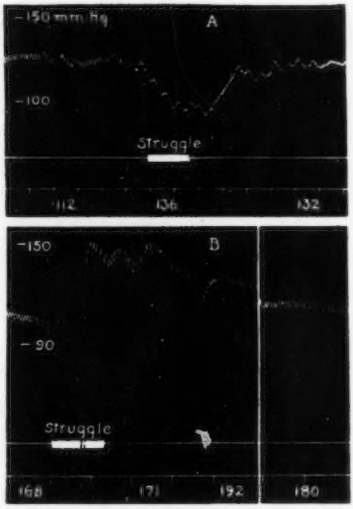

Fig. 1. Changes in blood pressure during struggle.

A. Completely sympathectomized animal, paralyzed and insensitive in its hind legs. No anesthesia. Blood pressure recorded from the femoral artery. Heart rate before struggle, 112 per minute; during struggle, 136; after, 132. In this and the following records the time is marked in 5 second intervals.

B. Normal animal. Spine transected at second lumbar vertebra 24 hours before, under ether. No anesthesia. Blood pressure recorded from the femoral artery. Heart rate before struggle, 168 per minute; during, 171; after, 180.

As a control the spinal cord of a normal animal was transected at the second lumbar vertebra under ether anesthesia and on the following day its blood pressure reactions to struggle were recorded. Figure 1 B shows the typical rise in pressure, which sharply contrasts with the fall of pressure in figure 1 A.

Vasomotor reflexes in animals under dial, with the sympathetic intact. Since we proposed using dial in our experiments it was necessary to prove that this anesthetic has no unusual effects on vasomotor reflexes. Records were obtained showing a fall in blood pressure on central stimulation of the left vagus with a tetanizing current of moderate intensity, a rise in pressure on central stimulation of the sciatic with a strong tetanizing current, and a fall in pressure, though of a less marked degree than that obtained with the vagus and depressor, on stimulating the central end of the saphenous with weak make and break single induction shocks at the rate of 4 per second. These standard reactions are so well known that no records are reproduced here.

Similar reactions were obtained even after section of both vagi, ligation of both adrenals and the administration of curare, as shown in figure 2, A,

B and C. The variations in the heart rate will be taken up in the discussion.

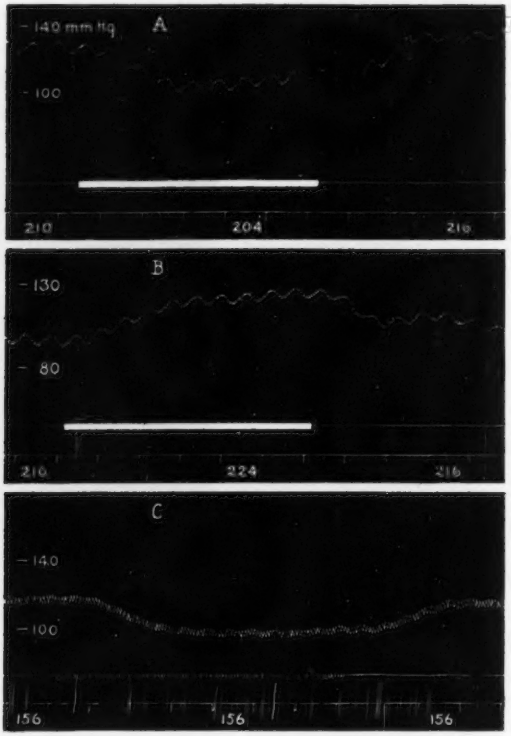

Fig. 2. Reflex changes in blood pressure in normal animal. *Dial anesthcia.* Both vagi cut and both adrenals ligated. Curare injected. Artificial respiration.

A. Fall in blood pressure on central stimulation of left vagus with tetanizing current. Secondary coil at 10 cm. Heart rate before stimulation, 210; during stimulation, 204; after, 216.

B. Rise in blood pressure on central stimulation of sciatic with tetanizing current. Secondary coil at 6 cm. Heart rate before stimulation, 216; during stimulation, 224; after 216.

C. Fall in blood pressure on central stimulation of the saphenous with make and break shocks at the rate of 4 per second. Secondary coil at 8 cm. Heart rate constant at 156.

Vasomotor reflexes in completely sympathectomized animals. Similar reflexes, varying only in degree, were obtained in sympathectomized preparations under dial even after section of both vagi and after the administration of curare.

Consistent vasomotor reflexes were somewhat more difficult to obtain before injection of curare since movements are associated with a fall in blood pressure, as mentioned above (see fig. 1 A). Figure 3 illustrates this difficulty. In figure 3 A the sciatic was stimulated with a strong tetanizing current in an animal with both vagi cut, but before giving curare. There was a *fall* in blood pressure associated with movements. In figure 3 B the median, stimulation of which has the same reflex effects as that of the sciatic on blood pressure, was similarly stimulated in another animal

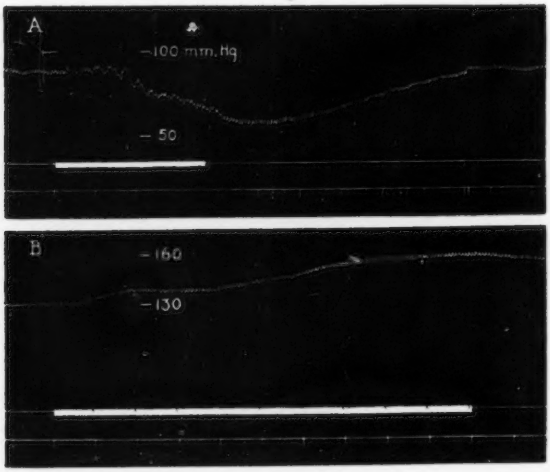

Fig. 3. Changes in blood pressure during central strong tetanization of afferent nerves in completely sympathectomized animals under dial.

A. Both vagi cut. Stimulation of the central end of cut sciatic produced movements, associated with a fall in blood pressure.

B. Both vagi cut. Stimulation of the central end of cut median nerve; only very slight local twitches. The blood pressure rose.

The heart rate did not vary in either case.

under identical conditions. There were no movements, and there was a *rise* in blood pressure.

If barely sufficient curare is injected to abolish visible movements, strong tetanization of the sciatic causes a rise, while moderate tetanization of the left vagus causes a fall in blood pressure, as shown in figure 4, A and B. This fall, though slight, was obtained consistently in all of our experiments. In view of the depressive influence of curare on vasodilation, a large fall was not to be expected. If too much curare is given, no vasomotor reflexes can be obtained even with the strongest stimulation.

Discussion. The fall in blood pressure which accompanies struggle or movements in sympathectomized animals (see fig. 1 A) can be due only to active vasodilation since all vasoconstrictor influences of the sympathico-adrenal mechanism have been removed, and there is an *increase* of the heart rate. In the normal animal struggle and movements are associated with rise in blood pressure (see fig. 1 B). Since the only difference between the two conditions is absence of the sympathico-adrenal system, the difference of effects in general arterial blood pressure must be due to that—i.e., to the absence of the constrictor influence of the sympathico-

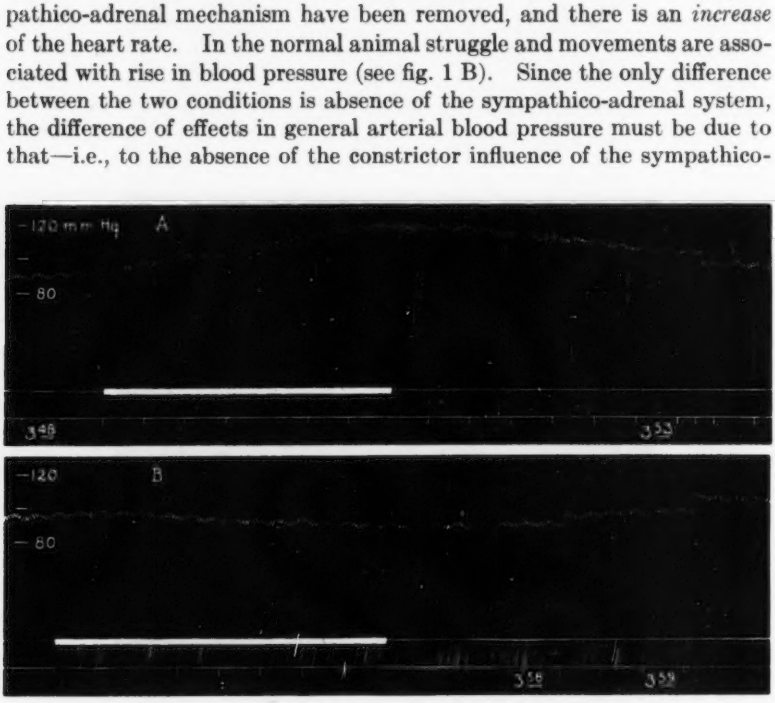

Fig. 4. Changes in blood pressure in completely sympathectomized animals with both vagi cut; under dial and injected with curare just sufficient to abolish all active movements.

A. Stimulation of central end of cut sciatic nerve; the blood pressure rose.

B. Stimulation of central end of cut left vagus and depressor nerves; the pressure fell.

The heart rate did not vary in either case.

adrenal system. It follows, therefore, that in the normal animal the rise of the general blood pressure results from a preponderance of the vasoconstrictor over the vasodilator influences.

It would seem, therefore, that a uniform reciprocal innervation of the vessels does not exist throughout the organism, since both constrictors and dilators are stimulated simultaneously. But if we consider that, although there is a predominance of the effects of constrictor tone, there occurs vascular dilation in active muscle, the idea of reciprocal innervation in the circulating system is preserved (Bayliss, 1908).

The fall of pressure occurring immediately at the start of the struggle shows that the vasodilation is due to nervous influence rather than to the local action of metabolites on the vessels.

In an animal in which all sympathetic vasoconstrictor impulses are excluded there is a definite vasodilator tone which can be increased reflexly by stimulating the depressor or by weak interrupted afferent stimulation of a sensory nerve, with a resulting fall in general arterial blood pressure. Vasodilator tone can likewise be decreased reflexly by strong tetanization of the sciatic or median nerve, with a resulting rise of pressure. That the heart rate does not account for these reflex changes in blood pressure, is proved by their occurrence in the absence of all known nervous and humoral influences affecting the heart, and by absence of any significant change in the heart rate (see fig. 4, A and B). Furthermore, any possible variations in blood pressure due to changes of skeletal muscle have been ruled out by repetition of the vascular reflexes after all movements had been abolished by curare. In these cases the possibility is excluded that the dilatation is due to muscular metabolites.

SUMMARY AND CONCLUSIONS

Vasomotor reflexes were investigated in completely sympathectomized cats under dial anesthesia, with the vagi cut, and both before and after the administration of curare in doses sufficient to abolish visible movement. Changes in blood pressure on struggle or movement were likewise studied in sympathectomized animals without anesthesia.

Dial anesthesia does not interfere with the production of pressor and depressor reflexes (see fig. 2, A, B and C).

In the sympathectomized animal without anesthesia and with vagi intact, struggle is associated with an immediate marked fall in blood pressure despite an increase of the heart rate; after struggle ceases the blood pressure promptly returns to its former level (see fig. 1 A). This fall in blood pressure is interpreted as due to an active vasodilation, of nervous origin.

In sympathectomized animals under dial anesthesia, and with vagi cut, stimulation of the sciatic often causes movements which are attended by a fall in blood pressure (see fig. 3 A); if movements do not occur there is a rise in blood pressure (see fig. 3 B).

In sympathectomized animals under dial and curare, with the vagi cut, both rises and falls in blood pressure may be obtained on stimulation of appropriate nerves, without changes in heart rate (see fig. 4, A and B).

It is concluded that there is a vasodilator system which can be reflexly stimulated or inhibited.

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THE FEATHER GERM AS INDICATOR FOR THYROID PREPARATIONS[1]

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Received for publication June 25, 1931

The possibilities of the feather germ as a rapid indicator for the female hormone—within forty-eight hours—have been reported rather recently (Juhn and Gustavson, 1930). Briefly, the method consists in the administration of a single injection during regeneration of male breast feathers of Brown Leghorn fowls and the subsequent daily preparation of feather germs. The young feathers are plucked, a median longitudinal incision made and the pulp resected. In this manner the first appearance of female modification occurring in the base of the feather germ may be determined, the time of persistence of the hormone in effective concentrations is recorded in the modified feather germ area formed daily and the time of passage of the hormone to subthreshold values due to excretion is given in the date of reversion of the feather germ to the male type.

It has now proved feasible to apply a similar method in the determination of the early response in the plumage to the administration of thyroxin and thyreoglobulin and to the persistence of these principles in effective concentrations subsequent to single injections.

This report is concerned exclusively with the possibilities of the reaction of the feather germ as a qualitative and quantitative indicator for the thyroid principle. A full review of the literature on thyroid administration in the fowl may therefore be omitted but reference should be made to the description by L. J. Cole and D. H. Reid (1924) of the accumulation of dark pigment in the definitive feather subsequent to thyroid feeding. These findings in the fully formed plumage have been confirmed and extended to include a similar response to thyroxin by H. B. Torrey (1926) and by B. M. Zawadowsky and Marie Rochlin (1928).

In the present study saddle feathers of capons from the Whitman Laboratory stock of Light Brown Leghorns were used. In this breed the saddle

[1] The expenses of this investigation were supported in part by the Committee for Research in Problems of Sex of the National Research Council, grant administered by F. R. Lillie; and by a special fund for biological research from the Rockefeller Foundation.

feathers are orange coloured and lacy in outline due the absence of marginal barbules. The injection of either thyroxin or thyreoglobulin causes the appearance of dark pigment granules in the basal, formative part of the feather germ in this region. This pigment deposit is associated with the formation of barbules normally lacking; this latter point will be neglected here and reference made exclusively to the induced dark pigment.

In all instances the reaction to single doses only were studied; these were administered by subcutaneous injection about the twentieth day of feather regeneration. The effects of thyroxin (Squibb's crystalline) were first tested. Doses of 0.5 mgm. cause the appearance within 48 hours of dark pigment in the feather germ base. This deposition of pigment continues for approximately 4 to 5 days after the injection, then reversion to the

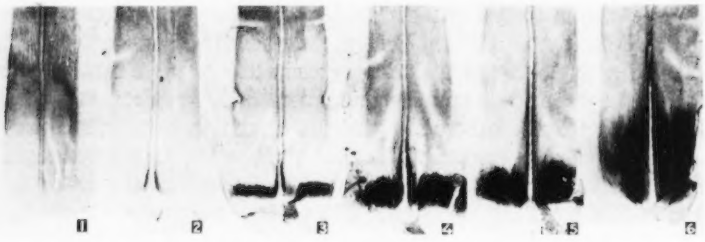

Plate 1. The response in the saddle feather germ of the Brown Leghorn capon to the adrainistration of a single, 1.5 mgm., dose of thyroxin.

1. Photograph of saddle feather germ prepared just previous to the injection of thyroxin.

2. Photograph of saddle feather germ prepared one day later showing the appearance of dark pigment in the feather germ base.

3, 4, 5. Photographs of saddle feather germs prepared 2, 3, and 4 days later showing the persistence of the reaction.

6. Photograph of saddle feather germ prepared five days later illustrating the reversion to the normal feather type.

normal feather type occurs. Heavier doses of thyroxin, 1.5 mgm. and 2.0 mgm. cause the formation of larger pigmented areas. Yet heavier doses, of 5.0 mgm., not only produce a further increase in the size of the induced pigmented areas but a very marked response is recorded earlier, within twenty-four hours after the injection.

There is, then, a correspondence between the concentration of the active principle and the degree of modification obtained as well as a correlation between the concentration and the time elapsing between the administration of the principle and its visible effect in the feather germ. The rate of disappearance of the principle from circulation is indicated in the gradual reversion of the feather germ to the normal type after the peak of effectiveness is passed.

The feather germ photographs in plate 1 illustrate the first appearance of the reaction to thyroxin and the time of its persistence. Figure 1 shows a saddle feather germ preparation made just previous to the injection of 1.5 mgm. thyroxin. Figure 2, a saddle feather germ prepared one day after the injection, shows the appearance of the reaction in the dark pigment deposited in the feather germ base. Figures 3, 4, and 5 illustrate feather germs prepared at daily intervals thereafter; the persistence of the response is manifest. Figure 6 illustrates the beginning of the reversion of the feather germ to the normal type five days after the administration of the single dose.

This marked response in the feather germ to thyroxin suggested the use of the reaction as a possible indicator for thyroid preparations. Thyreoglobulin was selected as test substance because Harrington (1930) has isolated thyroxin from it and Koch (1913) has shown that thyreoglobulin is as physiologically reactive as desiccated thyroid.

To single subcutaneous injections of thyreoglobulin the same general response in the feather germ was obtained as with thyroxin and also the same correlation between dosage and modified area size, and between dosage and time of the first visible response. However, with thyreoglobulin, the first visible response was slower in appearance than with thyroxin and the effective concentration was slower in reaching its maximum. The indications are that either absorption of the protein occurs more slowly or that it undergoes some change previous to becoming physiologically active. This will be studied further by intravenous injections. Also, the threshold of either substance is being determined in order to compare their effectiveness in terms of their iodine content.

The response obtained to thyroxin and thyreoglobulin seemed hardly attributable to the iodine contained in these compounds for only very small amounts of this substance were administered with the dosages used. Nevertheless both inorganic iodine and organic iodides were tested for their reaction in the feather germ. Injections of potassium iodide proved negative even though the dosage corresponded on the basis of the relative iodine to about 4 mgm. thyroxin or approximately eight times the lowest concentration tested. Methylene iodide, propyliodide and phenyl iodide were tested in concentrations equivalent to 100 times the iodine quantity present in 0.5 mgm. thyroxin. These proved negative although phenyl iodide has the same linkage between the benzene ring and the iodine as is found in the thyroxin molecule. Since all these last substances proved negative it seems that the positive results obtained with thyroxin and thyreoglobulin are not related to the iodine. However, two other compounds are to be tested shortly, di-iodotyrosine and des-iodothyroxin.

In so far as tissue specificities are concerned, the reaction is not obtained with either female or male hormone preparations. Extracts of liver, pan-

creas and spleen prepared in the same manner used for thyreoglobulin isolation have given equally negative results.

Our experiments show therefore that the feather germ reaction may be used as a qualitative indicator for the thyroid principle. Definite results can be obtained with small quantities and in a comparatively short time. It also offers possibilities as a quantitative indicator; its limits as a method for quantitative assays remain to be determined. For the present the following points may be enumerated from our findings; they are now subject of further investigation:

1. The response in the feather germ to either thyroxin or thyreoglobulin is roughly proportional to the dosage. Within limits, therefore, the width of the pigmented area in the base of the feather germ deposited day by day is an index of the circulating effective principle. 2. The response to either compound occurs much sooner with heavier doses, the time of its first appearance may thus be taken as an indication of concentration. 3. Since single injections give a permanent daily record of effectiveness for a number of days, the feather germ offers an instrument for the study of the cumulative action of spaced, repeated dosages. 4. The threshold of response of a series of birds may be determined. Using various dilutions of an unknown, it should be possible to roughly gauge the concentration from the last dilution giving a positive response.

SUMMARY

1. The feather germ reaction to thyroxin and thyreoglobulin as a qualitative test is described.

2. The reaction as an index of the time of effectiveness of single doses is discussed.

3. The possibilities of using the method quantitatively are suggested.

4. The test is negative with inorganic iodine and with the organic iodides so far tested.

5. The sex hormones and extracts of liver, pancreas and spleen are negative.

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THE RESPONSE OF SYMPATHECTOMIZED ANIMALS TO INSULIN

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Received for publication June 10, 1931

The data presented in this communication were obtained during a study of the action of endocrine extracts on animals surviving exclusion of sympathetic nerve impulses. The operation has been described by Cannon, Newton, Bright, Menkin and Moore (1929).

Considerable evidence has been adduced in recent years that the sympathico-adrenal mechanism plays an important part in the bodily defence against insulin hypoglycemia. Burn (1923) found that after administration of ergotoxin an animal became hypersensitive to insulin, due presumably to paralysis of the sympathetic nerves. Cannon, McIver and Bliss (1924) showed that as the blood sugar in the insulinized cat fell below a certain critical level the denervated heart beat with increasing frequency. At this time there was a retardation in the rate of fall of the glycemic level. After removal of both adrenal glands, or removal of one gland and denervation of the other, the cardiac acceleration did not occur; the fall of blood sugar was less retarded at the critical level, and convulsive seizures were induced sooner and with smaller doses. Britton, Geiling and Calvery (1928) were able to substantiate and to extend these observations. They reported that suppression of adrenin secretion (by destruction of the adrenal medulla or simple section of the nerves) resulted in a four- to tenfold increase in sensitivity to insulin; moreover adrenal-inactivated animals showed no increase in blood sugar after convulsions and no ability to recover spontaneously from insulin hypoglycemia. In line with these results are the findings of several workers, to the effect that adrenalectomized animals are hypersensitive to insulin. The general conclusion drawn from these experiments is that during insulin hypoglycemia there is an augmented medulliadrenal secretion, stimulated centrally or reflexly, and mediated by the sympathetic nervous system.

The present research had as its object a study of the reaction to insulin of animals in which the central connections of the sympathetic nerves had been interrupted and degeneration of the post-ganglionic fibres had

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occurred. In these animals both sympathetic chains were excised from the stellate ganglion to the ganglion impar in the pelvis. The adrenal glands were left intact, but these structures, along with the liver and skeletal muscles, were deprived of their sympathetic nervous connections. Thus was afforded a means of observing the effect of simple interruption of the nervous pathway on the response to insulin hypoglycemia.

At the time of use the animals (cats) had fully recovered from the acute operative effects, and were in excellent condition as regards maintenance of body weight and activity. Insulin was injected either subcutaneously, or into one of the marginal ear veins, in amounts mentioned below. Sugar content of ear-vein blood was measured by the method of Hagedorn and Jensen (1923) and in some cases by the micro-method of Folin (1928).

TABLE 1

The blood sugar of normal and of sympathectomized animals in the unanesthetized state

NORMAL		SYMPATHECTOMIZED	
Animal number	Blood sugar	Animal number	Blood sugar
	mgm. per cent		*mgm. per cent*
Cat 51	106–115	Cat 107	87–97
Cat 59	114–116	Cat 286	76–82
Cat 60	124–132	Cat 294	77–82
Cat 100	112–120	Cat 295	82–88
Cat 102	103–106	Cat 384	80
Cat 103	110–112	Cat 391	80
Cat 264	106	Cat 400	107–115
Cat 265	88–94	Cat 456	83–86
Cat 266	103–106	Dog X	81–83
Cat 267	136–138	Dog Y	86–88
Cat 269	116		
Cat 270	93–96		

Owing to the fact that the sympathectomy operations had been completed (under ether) some time previously, and since the removal of ear-vein blood as well as the injection of insulin are very simple procedures, it was possible to carry out the observations in non-anesthetized animals.

RESULTS. 1. *The blood sugar in sympathectomized animals.* In table 1 are figures comparing the sugar concentration in the blood of active normal and sympathectomized cats. It will be noted that, while the sugar content after sympathectomy is not outside of the normal range, it does not show the variation found in a series of normal cats. The more constant and lower blood-sugar content in the operated cat is probably due to absence of the characteristic response to emotional stimuli (cf. Britton, 1928). The figures for the sympathectomized cats are in agreement with those of Cannon, Newton, Bright, Menkin and Moore (*loc. cit.*).

2. *Effect of insulin.* Every experiment gave results similar to those obtained by previous workers after suppression of medulliadrenal secretion. Doses of insulin as small as 0.1 unit per kilogram lowered the blood sugar rapidly. In every instance except one typical hypoglycemic signs developed, and when restorative measures were not employed convulsions supervened. These convulsive fits were severe and of a marked tonic type, rather than the mixed or predominantly clonic seizures observed in normal animals. Normal cats, as is well known, exhibit a prolonged series of attacks, separated by intervals of partial recovery, and they often recover spontaneously when left to themselves. This has never been observed

TABLE 2

Data comparing the general effects of insulin on sypathectomized and normal cats

NORMAL				SYMPATHECTOMIZED			
Number	Insulin	Time onset First signs	Time onset Convulsion	Number	Insulin	Time onset First signs	Time onset Convulsion
	units kgm.	minutes after injection			*units kgm.*	minutes after injection	
297	6.0	98	115	456	0.4	58	
298	6.0	126	137	286	1.0	80	
298	6.0	*		286	1.0	38	53
299	3.0	183	190	294	0.5	65	
299	4.0	155		294	1.0	82	
299	4.0	95	100	295	0.7	25	30
300	4.0	100		295	0.4	72	
300	4.0	*		384	0.5	52	
495	5.0	105	110	384	0.3	62	72
497	5.0	185		107	0.5	55	
265	4.0	370		391	0.2	73	75
266	3.0	138	218	391	0.2	115	
				400	0.2	75	
				400	0.3	70	
				396	0.3	100	
				403	0.2	105	

* No signs in four hours.

in sympathectomized animals. After two or three short but severe seizures, respirations tended to stop and the heart slowed. In three experiments in which no effort was made to revive the animal, death followed quickly, without any evidence whatever of spontaneous recovery.

In table 2 are data comparing the general effects of insulin on sympathectomized and normal cats. It will be noted that with doses of insulin varying between 0.2 and 1 unit per kilogram all sympathectomized animals quickly developed signs of hypoglycemia. The normal animals, however, were able to withstand from 3 to 6 units per kilogram for a much longer time, and in two cases showed no outward signs of a low blood-sugar level.

Apart from the type of convulsive seizures, the hypoglycemic signs exhibited by denervated animals are similar to those manifested by the normal. Restlessness, polypnea, relaxation of sphincters, crying and salivation occurred as the blood sugar reached a low level. The polypnea was not so rapid as in the intact animal, but no special observations were made on this point. Salivation occurred in every sympathectomized cat, and in the writer's experience was more constantly observed, and more profuse, than in the normal hypoglycemic animal.

3. *The behavior of the blood sugar.* The increased susceptibility of sympathectomized animals to insulin was most clearly demonstrated by the behavior of the blood sugar. In figure 1 are shown typical insulin-sugar curves of sympathectomized and control animals. It will be noted that

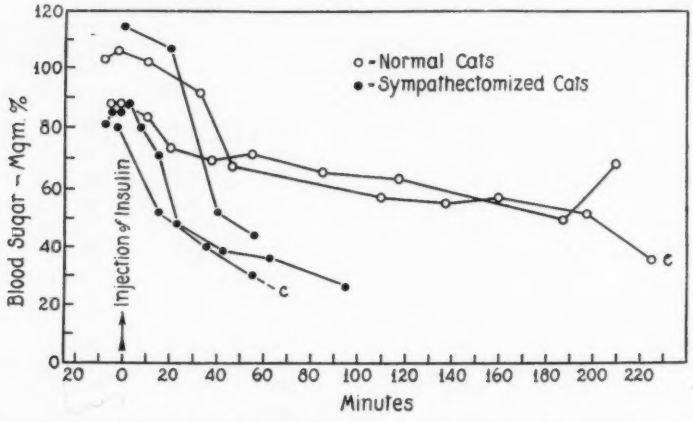

Fig. 1. Typical graphs of blood sugar in sympathectomized and control animals after insulin (cf. table 2). C indicates convulsion.

the former animals received from $\frac{1}{3}$ to $\frac{1}{1\frac{1}{2}}$ the amount of insulin given to the controls.

Two features are noteworthy in the sympathectomized cat: 1, whether the insulin be given subcutaneously or intravenously, the fall in blood sugar is rapid and fairly uniform; 2, at no time afterward is there evidence of spontaneous recovery of the blood-sugar level. The preliminary fall, i.e., the decrease until the critical level is reached, is not appreciably accelerated in the denervated animals; it has been pointed out by Macleod (1926) that this phase of insulin action is, within wide limits, independent of the size of the dose. The present results indicate that it is not influenced by the functioning of the sympathetic nervous system. But the secondary fall is markedly affected. In the normal cat this phase is characterized by a

distinct retardation in the rate of fall of the blood-sugar content. Even after the sugar concentration has reached a low level there often occurs a slow, spontaneous increase which may lead to complete recovery. In the denervated cats, however, the sugar curve throughout continues at a steep and uniform slope, and the fall remains unchecked until the convulsive level is reached and passed. Nor is there any marked increase observable after the convulsive seizures; the animals that died of hypoglycemia passed out at the lowest blood-sugar level noted in the course of the experiment. This precipitous, uncompensated fall occurred with doses of insulin that would scarcely affect the blood sugar of a normal animal.

4. *The occurrence of an initial hyperglycemia.* A brief yet marked increase in the blood sugar of some of the sympathectomized cats occurred very soon after an intravenous injection of insulin. Results of this type are illustrated in figure 2. In this respect the operated animals were not different from the normal. Does this preliminary rise coincide with a short period of hepatic glycogenolysis? If this were the case, then it would follow that hepatic glycogenolysis after insulin is not dependent only upon the activity of the sympathico-adrenal mechanism. The physiological significance of this phenomenon is not clear. Geiling and DeLawder (1930) were able to eliminate this phase of insulin activity by using a purified extract, and concluded that the initial hyperglycemia obtained with certain commercial preparations is not an intrinsic property of the pure hormone. The above observations, therefore, do not necessarily mean that insulin can release liver glycogen after sympathico-adrenal inactivation.

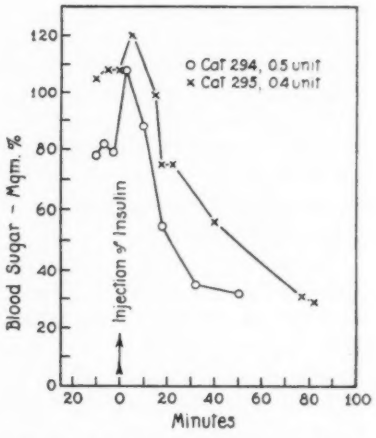

Fig. 2. A brief initial increase of blood sugar after injection of insulin.

5. *Recovery after adrenaline or glucose administration.* A certain method of bringing about recovery from all stages of hypoglycemia was by the subcutaneous injection of adrenaline solution. Even a single intravenous injection was found adequate in three cases; in other instances such doses produced a marked temporary improvement, but after 15 to 20 minutes the animals were again in a state of collapse. The subcutaneous injections always brought about a complete and permanent restitution to normal.

Glucose, given subcutaneously or intravenously, even in small amounts, had a permanent restorative effect. The smallest quantities found efficacious were 1 gram per animal, in twenty per cent solution. Most of the cats received from 2 to 8 grams, and recovered promptly. Subsequent relapses, once glucose had been administered, were not observed. These results are in marked contrast to the course of events in the intact hypoglycemic cat, in which much larger quantities of glucose are required to accelerate recovery. Further, the normal cat may exhibit several relapses, necessitating more sugar injections, before complete recovery is established.

Discussion. Cannon (1929) has reviewed the factors which make for the constancy of the glycemic level, and has pointed out the primary rôle which the sympathico-adrenal mechanism subserves in this regard. Through this agency factors are set at work to counteract any marked reduction in the blood-sugar content. Its function is evident during two stages of insulin activity: 1, when the blood-sugar level has reached a certain critical mark (about 70 mgm. per 100 cc. in the unanesthetized cat); 2, during generalized hypoglycemic convulsions. This function of the sympathetic may thus be considered as an emergency action, called upon to protect the body against the dangerous effects of a very low blood-sugar concentration.

The present results, obtained under rather favorable experimental conditions, furnish additional evidence in support of this contention. Cats deprived of central sympathetic innervation—while the adrenal glands and the liver are left intact—are rendered hypersensitive to insulin and remain so permanently. In such animals, once insulin has been injected and the blood sugar begins to fall, there appears no sign of the glycemic effect of sympathico-adrenal activity, either at the critical level or during convulsions. Elliott (1912) has shown that the adrenal gland contains normal quantities of adrenin even after degenerative section of the splanchnic nerves. It is therefore apparent that an essential rôle in the sympathico-adrenal protection against hypoglycemia is mediated by sympathetic nerve impulses.

Under ordinary conditions of existence, these animals show no evidence of grave impairment of the blood-sugar regulating mechanism. Thus spontaneous hypoglycemia has never been observed in sympathectomized cats, although, as has been stated, the basal blood-sugar level is relatively low as compared with active control animals. It would seem that the insulin secreted in the course of ordinary metabolism can be well handled by factors that do not require sympathetic nervous impulses. One may, indeed, suppose that in these animals the production of insulin occurs at a physiologically minimal rate. What would happen if any excess were left circulating in the blood stream is obvious. From this inference, and the fact that recovery from hypoglycemia may be effected in these animals

by the injection of as little as one gram of glucose, it may be concluded that the insulin-carbohydrate balance is very delicately adjusted, and maintained on a narrow margin. The fact that epinephrine abolishes the hypoglycemia indicates that the hormonal mechanism for increasing the blood-sugar level is not impaired in sympathectomized animals—whether it functions by releasing liver glycogen, or inhibiting peripheral sugar utilization (Cori, 1931).

It is noteworthy that the four sympathectomized animals which had a convulsion, as recorded in table 2, received respectively only 0.2, 0.3, 0.7 and 1.0 unit of insulin per kilo and that the convulsion appeared from 30 to 75 minutes after the injection. In six different animals, with adrenals inactivated but with the sympathetic system otherwise intact, reported by Cannon, McIver, and Bliss (1924), that received uniformly a larger dose (2.0 units of insulin per kilo), the convulsion appeared at a considerably later time—67, 70, 78, 90, 95 and 100 minutes after the injection. The liver was innervated by one set of splanchnic fibres in these animals and that condition may have been favorable, though the evidence for efficacy of the nervous control of glycogenolysis is not strong (cf. Bulatao and Cannon, 1925; Britton, 1928). It seems quite possible that in the reaction of the sympathetic system to hypoglycemia in these animals the setting free of sympathin from all parts of the body might have been serviceable in mobilizing sugar, as adrenin does, and thereby helping to protect the organism against hypoglycemic convulsions.

The occurrence of such hypoglycemic manifestations as muscular twitching, convulsions, polypnea and salivation in sympathectomized cats indicates that, if these phenomena are of nervous origin, their occurrence must be ascribed to other than central sympathetic impulses. The increased salivation is consistent with the claim of Ponirovsky and Ischunina (1929) that insulin is a powerful stimulant of parasympathetic salivary secretion.

SUMMARY AND CONCLUSIONS

Bilateral extirpation of the sympathetic nerve chains renders cats permanently hypersensitive to injected insulin (see fig. 1 and table 2). Even after small doses all the signs of hypoglycemia quickly supervene; these include polypnea, salivation, relaxation of sphincters, and strong tonic convulsions.

The blood-sugar curves after insulin show a rapid and precipitous decline, with no evidence of spontaneous recovery. Restitution to normal can be effected by injection of small amounts of epinephrine or of glucose.

Inasmuch as these animals never develop hypoglycemic symptoms under natural conditions, it is inferred that the physiological insulin secretion in these cats is always at a minimum, both in rate and quantity.

In some instances after intravenous injection there is a brief initial hyperglycemia (see fig. 2); its significance is unknown.

This problem was suggested by Prof. W. B. Cannon, to whom I am indebted for valuable advice and criticism.

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BIOELECTRICAL MEASUREMENTS ON AMOEBAE

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Received for publication May 11, 1931

The various theories for the interpretation of bioelectric phenomena generally postulate that potential differences must be present at the membranes of the single cellular elements. Crile (1) has suggested that the effect of various stimulating or injuring agents, physical or chemical, must be connected with changes in the bioelectric potential differences of living systems and that such changes must invariably be connected with corresponding electrical changes in living tissues (2) and single cells.

A great advance would be made in our knowledge of bioelectric phenomena if it were possible to make measurements of the single cell. Osterhout (3) recognized the need for such a study and his experiments on Valonia (4) and Nitella (5) furnish findings concerning the current of injury in single cells (6).

The development of micromanipulative technic (5) has enabled us to carry out similar experiments on very small unicellular organisms. This paper describes bioelectric phenomena which may be observed in amoebae. The experiments were carried out with the following aims:

1. To ascertain whether there is a certain and definite Potential Difference (P.D.) between any point inside the amoeba and the culture medium. (The term "culture medium" as used here indicates the solution in which the living amoeba is suspended.)

2. To ascertain whether or not this possible P.D. is changed by changing the chemical composition of the culture medium.

3. To ascertain whether there is any relation between the viability of the amoeba in a certain medium and the P.D. between the amoeba and that medium.

In order to make these measurements it was necessary to connect reversible electrodes to the two points between which the P.D. was to be measured. One electrode was inserted into the amoeba. (This does not influence its viability to any great extent.) The other electrode was placed in the culture medium, the end of each of the two reversible electrodes being filled with culture medium. This chain can be illustrated by the following scheme:

inside amoeba outside
electrode / culture medium / protoplasm membrane / culture medium / electrode

This arrangement is analogous to that used in the different measurements of injury potential made on larger physiological objects (surviving tissues, fruits, etc.) and is essentially the same as Osterhout's scheme and working hypothesis for the Valonia:

sap / *X* / *W* / *Y* / sap

in which *X*, *W*, *Y* represent the different suggested layers of the protoplasm.

According to the measurements of Ettisch and Péterfi, (7), (8) no potential differences exist in the inside of a unicellular organism (amoeba) within the limits of their method. The constant streaming of the protoplasm would indicate that as far as the inside of the amoeba is concerned, there are no layers present, but there is still the possibility that there might exist a layerlike arrangement close to the membrane of the amoeba, the thickness of which might be beyond the limits of the measuring technic of Ettisch and Péterfi.

The system mentioned above—electrode / culture medium / protoplasm amoeba membrane / culture medium / electrode—would show the P.D. which exists between the amoeba and its normal environment. If the outside culture medium (the drop in which the amoeba is placed on the microscopic slide) is changed by the addition of a certain electrolyte or other chemical substance, the arrangement becomes asymmetrical:

electrode / culture medium / amoeba / culture medium + solution / electrode

This arrangement will make it possible to measure the change of the P.D. on the amoeba and a small but negligible diffusion potential at the electrode. The experimental method which we have used is similar to the one described by Ettisch and Péterfi (6) except that the electrodes were altered.

EXPERIMENTAL METHOD. The apparatus consists of the microelectrodes, micromanipulator and electrometer

1. Microelectrodes. The microelectrode which was used is a combination of electrode and microinjection needle, so arranged that the end of the electrode can be filled with different solutions. A piece of glass tubing $\frac{1}{8}$ inch in diameter is connected with another short piece, of larger diameter, to form a T-piece (A). Into the short end a syringe of 2.5 to 3 cc. capacity is cemented with de Khotinsky cement. One end of the T-tube is cut about 1 inch long, while the other is drawn out into a capillary, leaving a shaft about 2 inches long. The capillary end is drawn out above a microburner to form a micropipette. The other end of the tube is closed with a filter-paper plug saturated with normal KCl solution, and the plug is pressed down close to the T-division. Some silver chloride is placed above the filter-paper and the tube is closed by cementing into it a silver wire coated with silver chloride. The whole electrode is then filled with normal

KCl solution, the solution being forced up with great precaution so as to exclude any air bubbles. By applying mechanical pressure to the syringe the KCl can be forced into the capillary. When the electrodes are ready for use they are mounted on the micromanipulator. The syringes are fixed vertically on the micromanipulator, the capillaries making an angle of about 60 degrees with the vertical line. To test the electrodes the ends of the capillaries are first dipped into a drop of nKCl and are then connected to the quadrant electrometer. There should be absolutely no P.D. between the two electrodes if they are filled with the same KCl solution.

2. The micromanipulator is one of the types devised by Prof. Robert Chambers (9). The microscope objective A 8 has a working distance of 7 mm. which is quite sufficient. We used two different eyepieces—an ×5 eyepiece for adjusting the electrodes and for looking over a larger field, and an ×15 eyepiece for the experiments.

The amoeba was placed on a slide which was mounted on the moving stage of the microscope. No wet-chamber was used and the amoeba was placed on the slide in a drop of culture medium. Meanwhile the tips of the micropipettes had been immersed in a drop of n/10 KCl placed on the same slide. The P.D. between the two electrodes, which naturally should be zero, was measured in this drop. Next the electrodes were short-circuited and the substage moved so that the tips of the electrodes reached into the drop, in which was the amoeba. The electrodes were filled with the solution in the drop by means of the syringe. The short-circuiting was then disconnected and the P.D. between the electrodes—which should again be zero, was measured. The electrodes were moved near the amoeba and one of them was inserted into the cytoplasm, while the other was placed about 20–50 microns away from the amoeba. The P.D. was measured, the system being as follows:

$$\text{electrode / culture medium / } \overbrace{\text{protoplasm membrane}}^{\text{amoeba}} \text{ / culture medium / electrode}$$

After the P.D. had been measured, the solution in which the amoeba was to be tested was added and the change in the P.D. was observed, together with the physiological reaction of the amoeba. When the experiment was finished, the electrode was removed from the amoeba and a measurement was again made to make sure that there was no P.D. between the electrodes.

When the cytoplasm of the amoeba jellified during the experiment, it sometimes happened that parts of the amoeba still adhered to the electrode or clogged it up. In this case some of the solution filling up the micro-electrodes was gently pressed out and the tip of the needle freed.

The electrometer used is of the quadrant type. The suspension wire was

selected so that the instrument recorded about 1.0 to 1.5 millivolt in response to a deflection of one millimeter on the scale. The whole micromanipulator was shielded with a wire cage to protect it, and the wires connecting the microelectrodes with the quadrant electrometer were shielded. The wires were carefully insulated. The electrical connections are shown in figure 1. The sensitivity of the measurements depends on the accuracy of the readings on the scale. This was divided into millimeters and half millimeters could be estimated. A deflection of half a millimeter would

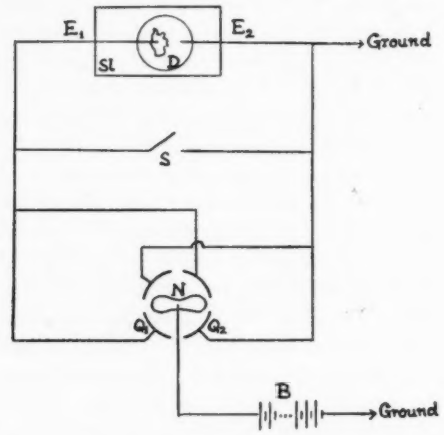

Fig. 1. Diagram showing the electrical connections. The needle N is charged by battery B. The quadrants Q_1 and Q_2 are connected to the electrodes E_1 and E_2. The switch S permits short-circuiting of the electrodes. One of the electrodes is dipped into a drop of culture medium D placed on the slide Sl. The other electrode is inserted into the amoeba.

correspond approximately to half a millivolt, which is the upper limit of possible error.

EXPERIMENTAL RESULTS. *1. P.D. of "injury" in the normal culture medium.* The P.D. measured in the arrangement:

Electrode / culture medium / amoeba / culture medium / electrode

could be compared with the "injury P.D." (or "current of injury") because the amoeba must be injured in order to introduce the electrode. It seems to be questionable whether or not there is a continuous injury after the introduction of the electrode. According to the experiments of Chambers and Reznikoff the injection of distilled water or of a very dilute salt solution is less injurious to the amoeba than is the injection of more concentrated salt solutions. That is why it seemed advisable to fill the end of the micro-

needle with the culture medium and introduce that into the amoeba, rather than to introduce the electrode filled with KCl solution, which even in a n/10 solution might change the electrolytes present in the amoeba because of the diffusion of some of the KCl out of the electrode.

In the course of very many experiments it was found that there is a definite measurable P.D. The cytoplasm was negative, the culture medium positive. This result was obtained at various times with different electrodes and with different stocks of amoebae. The average P.D. measured was around 15 millivolts. Sometimes it was as low as 10 millivolts and almost never higher than 20 millivolts.

Ettisch (10) reports that in his experiments the P.D. was temporarily changed by the addition of small salt crystals to the solution. In our experiments instead of adding small crystals, a salt solution of known concentration was added to the drop containing the amoeba by means of a micropipette. The effect of the salt solution is necessarily a "concentration P.D." effect.

2. "Concentration P.D." in various salt solutions. The effect of distilled water was an immediate increase of the P.D. When the amoeba was washed several times with distilled water the P.D. increased to as much as 40 millivolts. This increase was accompanied by an increase in the protoplasmic motility.

The effect of various salts can be summarized as follows: Very dilute solutions either have no effect on the P.D. or increase it. Solutions of higher concentration lower the P.D. temporarily but in a minute or two the P.D. again attains the original value and the amoeba seems to be as lively as before. This finding is in complete accordance with the work of Ettisch and Péterfi. More concentrated solutions change the P.D. permanently, lowering it or even reversing the charge. Concentrated solutions reduce the P.D. permanently to zero. Very often a gradual fall of the P.D. could be observed, the zero point being reached some time later. In some cases the recovery was rather slow or was incomplete. The experimental results given in table 1 are the averages from at least ten measurements in each case.

Effect of KCl. A solution of KCl with a concentration of n/200 hardly changed the P.D. There was a slight decrease of one or two millivolts, but the original value was regained in one minute or less. With n/50 KCl a permanent decrease of about 5 millivolts was observed. Increasing concentrations decreased the P.D. more and more. With n/30 KCl the zero P.D. was reached in five to ten minutes, while a solution of n/20 or more concentrated solutions killed the amoeba more rapidly. In all cases when the P.D. became zero permanently the amoeba rounded up. With the n/20 or more concentrated solutions the death of the amoeba resulted almost immediately.

Effect of NaCl. NaCl in n/300 solution had no effect. A decreased P.D. could be observed with a solution of n/160, but it was maintained for only one or two minutes. In n/80 NaCl there was an immediate decrease of 10 millivolts followed by a partial recovery. In n/20 solution the decrease was permanent although the zero point was not reached. In n/5 solution the decrease of the P.D. to zero was rapid and permanent and the amoeba died.

Effect of HCl. A solution of HCl of pH 4 gradually decreased the P.D., the zero point being reached in about 14 to 20 minutes. In a solution of pH 3 the zero point was reached in a few minutes and in all cases the amoeba perished.

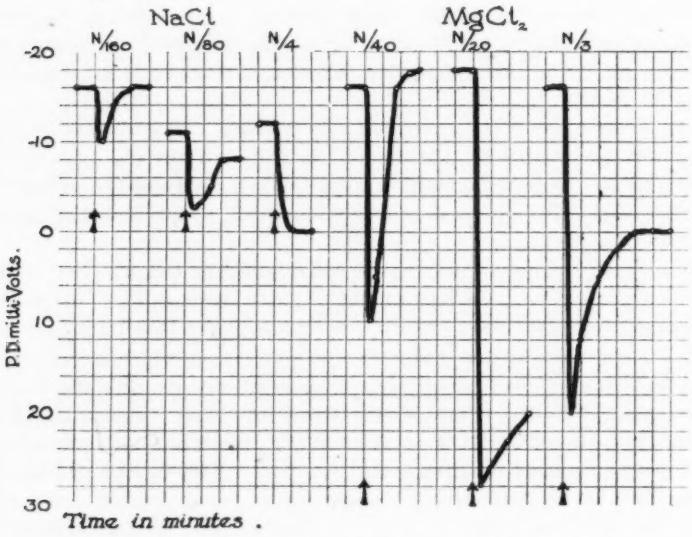

Fig. 2. The effect of NaCl and $MgCl_2$ on the P.D. of the amoeba

Effect of $CaCl_2$. Solutions of $CaCl_2$ more dilute than n/40 had a short temporary influence only, the original P.D. being completely recovered. In solutions of n/20 *the P.D. was reversed*, that is, the cytoplasm became positive against the solution. This reversed charge was measurable immediately after the $CaCl_2$ solution was added and it was possible to follow the change of the P.D. as it was indicated by the marking light reflected from the electrometer to the scale. In n/20 solution the amoeba retained its viability for some time and the P.D. remained reversed also. In n/5 solution the reverse of the charge was even more marked. With more concentrated solutions the reversed charge very soon dropped to zero and the amoeba died.

Effect of $MgCl_2$. In solutions of $MgCl_2$ more diluted than n/40 there was a lowering or even a reversal of the P.D. with more or less complete recovery of the original P.D. In n/40 solution the original charge of −16 millivolts was changed to +10 millivolts and recovery followed in 3 minutes. In n/30 solution a slow and only partial recovery could be observed. In n/3 $MgCl_2$ the charge was reversed and gradually reached zero in 8 minutes.

Effect of $NaHCO_3$. In very dilute solutions of $NaHCO_3$ corresponding to pH 8 to pH 10 there was a slight temporary increase of the P.D. which returned to its normal value in from five to ten minutes. In more concentrated solutions there was first a temporary and later a permanent decrease in the P.D. Solutions with a concentration of n/50 decreased the P.D. immediately and the amoeba perished.

TABLE 1

The effect of salt solutions on the P. D. of amoebae

SALT CONCENTRATION	IMMEDIATE EFFECT ON THE P. D.	LATER EFFECT ON THE P. D.	AMOEBA AFTER 10 MINUTES
KCl n/200	Small decrease	Recovery in one minute	Alive
KCl n/50	Medium decrease	No recovery	Alive
KCl n/20	Complete decrease	Zero in few minutes	Dead
NaCl n/160	Small decrease	Recovery in one minute	Alive
NaCl n/80	Medium decrease	Partial recovery	Alive
NaCl n/20	Large decrease	No recovery	Alive
NaCl n/5	Complete decrease	Zero	Dead
HCl pH 4	Gradual decrease	Zero in 15–20 minutes	Dead
HCl pH 3	Gradual decrease	Zero in few minutes	Dead
$CaCl_2$ n/40	Medium decrease	Recovery	Alive
$CaCl_2$ n/20	Reversed P. D.	Small recovery	Alive
$CaCl_2$ n/5	Reversed P. D.	No recovery	Alive
$CaCl_2$ n/3	Reversed P. D.	To zero in few minutes	Dead
$MgCl_2$ n/40	Reversed P. D.	Recovery	Alive
$MgCl_2$ n/20	Reversed P. D.	Partial recovery	Alive
$MgCl_2$ n/3	Reversed P. D.	To zero in few minutes	Dead
$NaHCO_3$ pH 8–10	Increase of P. D.	Returning to original	Alive
$NaHCO_3$ n/100	Gradual decrease	To zero in few minutes	Dead

The effects of the solutions of NaCl and of $MgCl_2$ are indicated in figure 2. The arrow marks the moment of the addition of the salt solution. The P.D. in millivolts and the time in minutes are the coördinates. In n/160 NaCl (fig. 2) there is a decrease of one minute's duration, with complete recovery. In n/80 solution the recovery is only partial. In n/4 solution the drop of the P.D. to zero is immediate and associated with the death of the amoeba. Figure 2 shows the remarkable *reversal of the P.D.* on the addition of $MgCl_2$. On the addition of n/50 solution there is

still complete recovery. With n/20 solution the recovery is incomplete and the positive charge is retained, while in n/3 solution the P.D. is reversed first, but then it gradually becomes zero with the death of the amoeba. The dots mark the actual measurements, which were taken every minute, the time being controlled very exactly. The results of the experiments described above are summarized in table 1.

Discussion. The viability of the amoeba in the various salt solutions as indicated by the experiments described above is in complete accordance with the results published by Chambers and Reznikoff. They report that the following salt concentrations are lethal in less than one hour: n/104 KCl, n/6.5 NaCl, while for $CaCl_2$ and $MgCl_2$ concentrations of more than n/6.5 are not lethal in one hour. As the single experiments on the P.D. of the amoeba were not carried on for longer than 20 minutes, the lethal doses were somewhat higher for the 20 minute period, which of course was to be expected.

The marked coincidence of the viability of the amoeba with its electrical P.D. is striking. The P.D. reaches its highest value in very dilute salt solutions (distilled water) and as the salt concentration increases, the P.D. decreases. At first the change is followed by a recovery, but with more concentrated solutions it leads to a total drop of the P.D. and death of the amoeba. The electrobiological importance of this finding is very significant when compared with the large number of results obtained from measurements of the P.D. of surviving tissues (muscle), etc. The extreme thinness of the membrane of the amoeba of course makes it possible that by diffusion the salts can penetrate very rapidly through it and in this way their biological activity is revealed more quickly and truly than in muscle, etc.

The measurements of the velocity of eggs, bacteria, etc., measured by cataphoresis and the charge calculated therefrom show a certain analogy with the direct measurement of the amoebae. In both cases there is a reversal of the charge with certain concentrations of $CaCl_2$ and $MgCl_2$.

SUMMARY

The P.D. of amoebae was measured with microelectrodes. There was found to be a definite P.D. between the interior of the amoeba and its exterior culture medium. The cytoplasm is negative, the culture medium positive.

This P.D. is changed by the addition of salt solutions. Very dilute salt solutions either have no effect on the P.D. or increase it. Less dilute solutions exert a temporary change with complete recovery. More concentrated solutions decrease the P.D. and the recovery is only partial. Even more concentrated solutions decrease the P.D. more or less rapidly to zero and the amoeba dies.

In certain concentrations $CaCl_2$ and $MgCl_2$ reverse the P.D. In more dilute solutions there is a recovery of the original P.D. but in more concentrated solutions the reversed charge remains and the amoeba retains its viability. In still more concentrated solutions the reversed P.D. gradually decreases to zero, and the amoeba dies.

The striking parallelism between the P.D. and the viability of the amoeba is evident.

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EVIDENCE FROM THE ALCOHOL BLOCK METHOD ON THE FREQUENCY OF MOTOR NERVE IMPULSES IN THE FLEXION REFLEX

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Received for publication June 12, 1931

It has been established by the work of Adrian (1913) and others that a region of partial narcosis in a nerve trunk will block a subnormal impulse set up in the relative refractory period following a previous impulse, even while the narcosis is inadequate to block a full-sized impulse evoked in the rested nerve. For example, when a mammalian motor nerve is subjected to a dilute alcohol solution, e.g., from 6 per cent to 12 per cent, for a distance of about 1 cm., after a lapse of a few minutes a stage is reached in which the full-sized impulse set up by a single stimulus will traverse the narcotized region and evoke a muscle response, whereas a second impulse set up within 2.5σ after the first will be totally blocked by the narcotic. When the narcosis has just reached a depth sufficient to block an impulse set up 2.5σ after the previous impulse, it will still permit the passage of an impulse set up 3σ after the first, for the latter impulse, although subnormal, is nevertheless more nearly full-sized than that set up after the briefer interval of 2.5σ. In this way the partial narcosis which just suffices to extinguish a second impulse has been used as a measure of the degree of subnormality of the impulses which are blocked, and therefore, by inference, as a measure of the frequency of those impulses.

Using this method, Forbes and Olmsted (1925) attempted to determine the frequency of motor neurone discharge in the crossed extension reflex in the decerebrate cat. An alcohol block was applied to the popliteal nerve, which innervates the gastrocnemius, an extensor of the ankle. As the narcosis became deeper, the crossed extension reflex was evoked at intervals, and in alternation with reflex stimulation, single stimuli were applied to the motor nerve proximal to the narcotic block. It was shown that the reflex responses of the muscle declined considerably as the narcosis deepened, at a time when the responses to single motor nerve stimuli were only slightly impaired. From this it was inferred that a majority of the motor-nerve impulses involved in the crossed extension reflex were subnormal and were therefore set up during the relative refractory period following the preceding impulses. Control experiments with similar narcotization of a

motor nerve, stimulated alternately with single stimuli and with successive stimuli of accurately determined intervals, showed that at approximately the same stage of narcosis at which the reflex had shown marked decrease, second impulses, set up at about 3σ after the first, were blocked in a majority of the motor nerve fibers. From these observations the inference was drawn that the frequency of motor-nerve impulses in the crossed extension reflex was not less than about 300 per second. It will presently appear that this inference must be revised; we shall return to this point after describing our observations.

In order to compare the flexion reflex with the crossed extension reflex in this respect, we have performed similar experiments, using the peroneal nerve and the tibialis anticus muscle as an indicator, and evoking the flexion reflex by stimulation of the popliteal nerve in the same leg. We have made our control of the apparent frequency more direct than that which was used in the observations of Forbes and Olmsted on the crossed extension reflex, by applying both single and double stimuli to the motor nerve in alternation with the reflex stimulation. The contraction of the muscle being recorded on a smoked drum, it was easy to observe by the presence or by the absence of summated contractions in the case of the double stimuli whether or not the second nerve impulse was getting through the block. Simultaneously the action currents of the muscle were recorded with a string galvanometer. This was done both in the case of reflex and of motor nerve stimulation.

Before performing our experiments on the flexion reflex, we made a series of preliminary control experiments to determine whether, under the conditions of our experiment, the second impulse set up during the relative refractory period following the first was blocked in the narcotized area because of being subnormal in size or because, on reaching the narcotized area, it encountered the absolute refractory period at that point, due to the prolongation of refractory period under narcosis, described by Kato (1924). This distinction, anticipated at the time of our experiments, has since been clearly analyzed by Kato and his collaborators (1929). They showed that a necessary condition for the phenomenon of Wedensky inhibition is that the second impulse shall fall in the relative refractory period of the narcotized region. If the second impulse follows the first by so short an interval that it arrives in the absolute refractory period of the narcotized region it produces no after-effect, and a third impulse following the second by a similar interval, will find the narcotized nerve sufficiently recovered from the first impulse to transmit it without block. Therefore, the result of a series of impulses set up with this frequency will be that every other impulse will pass the block, and instead of the initial twitch followed by relaxation, characteristic of the Wedensky effect, there will be a tetanic contraction of the muscle. Under these conditions, the failure of the

second impulse of a pair to reach the muscle is evidence, not that it is subnormal in size, but merely that it arrives too early, i.e., during the absolute refractory period of the narcotized region when it can set up no impulse at all in that region. We, therefore, arranged a Lucas pendulum to deliver three impulses at intervals of from 2.5 to 3σ. With 12 per cent alcohol in the narcotizing chamber on the peroneal nerve of the cat, we compared the contractions evoked by stimulus 1 alone, stimuli 1 and 2, and stimuli 1, 2 and 3 as the narcosis progressed, all stimuli being supramaximal. If the conditions of our experiment were right for Wedensky inhibition, (i.e.,

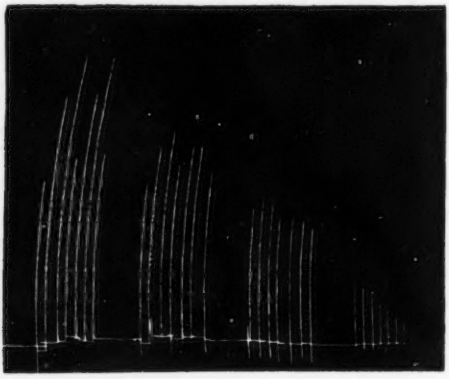

Fig. 1. Test of properties of block, showing that the third impulse is blocked as early in narcosis as the second. In each group the three stimuli are applied as follows: 1; 1,2; 1,2,3; 1; 1,2; 1,2,3; 1. In the third group where summated contraction with two stimuli is still present the third stimulus gives no additional summation. January 10, 1927.

subnormal impulses blocked in the narcotized area) summated contraction in response to all three stimuli should disappear at the same time as summated contraction in response to stimuli 1 and 2. If, on the other hand, conditions were right for alternation, (i.e., if the second impulse were blocked because of the prolonged absolute refractory period in the narcotized region) we should find evidence that the third impulse passed through the block. This condition would be revealed by persistence of summated contraction in response to all three stimuli after it had disappeared on application of stimuli 1 and 2.

We carried out seven such control experiments on three preparations, with intervals of 2.5 to 3σ, and in every case summation with three stimuli disappeared as soon as it did with two. There was no evidence of the transmission of alternate impulses, and therefore the condition appeared

to be one of a true Wedensky effect. An example of this control experiment is shown in figure 1.

The narcotizing chamber used was 12 mm. long. In this way we made sure of a region of uniform narcosis, which Kato (1924) has shown to be obtainable only when the chamber is at least 6 mm. long.

METHOD. In our experiments on the flexion reflex the procedure was as follows. The animal (cat) was decerebrated by the guillotine method, under deep ether anesthesia. The right sciatic nerve was exposed and the popliteal and peroneal branches dissected apart from hip to knee. The peroneal branch, being the motor nerve to the tibialis anticus muscle, which participates in the flexion reflex, was left intact. The popliteal nerve was severed at the knee, and used as the afferent nerve to evoke the reflex. The hamstring nerve was cut in order to eliminate disturbing motion, and for the same reason most of the muscles were cut away from the hip joint. The femur and tibia were both firmly clamped. Contractions of the tibialis anticus muscle were recorded by means of a thread attached to the foot approximately midway between the heel and the toes and attached at the other end to a light, aluminum, bell-crank lever, which recorded on a smoked drum. For the purpose of recording action currents, twine wicks from agar electrodes were stitched lightly to the surface of the tibialis anticus muscle, through small windows cut in the skin. These were connected with a Hindle string galvanometer.

Stimulating electrodes in a glass shield were applied to the popliteal nerve, connected with a pair of balanced coils (cf. Bishop, Erlanger and Gasser, 1926) whose primary circuit was interrupted by a rotary interrupter. The frequency of the reflex stimuli thus delivered was approximately 300 per second. Another pair of stimulating electrodes was applied to the peroneal nerve near the hip in a glass shield with a slot which rendered possible its application to the uncut nerve. These electrodes were connected with the secondary coils of two inductoriums in parallel. Their primary circuits were connected with two keys of a Lucas pendulum, whereby the break shocks could be delivered at any desired interval. Between the stimulating electrodes on the motor nerve and the tibialis anticus muscle was a 12 mm. narcotizing chamber through which the nerve was led. The arrangement of apparatus is shown in figure 2.

Before narcotization was begun, a series of reflex responses was recorded, interspersed with control series, in which single and double stimuli alternately were applied to the motor nerve, and whereby the degree of summated contraction resulting from the second of a pair of nerve impulses was determined. Each control test consisted of a single stimulus, then two stimuli with an interval of 2.5σ, and then another single stimulus; a rest of two or three seconds was allowed after each stimulus or pair of stim-

uli in a series. The strengths of stimuli were so chosen that both the first and second stimulus of the control pair were supramaximal.

After a preliminary series of records was made, Ringer's solution containing 10 per cent to 12 per cent alcohol warmed to the temperature of the nerve (about 30°C.), was introduced into the narcotizing chamber. As the narcosis progressed, similar observations were continued in alternation, until the nerve impulses were totally blocked by the narcotic. In some experiments as soon as summated contraction with a pair of stimuli

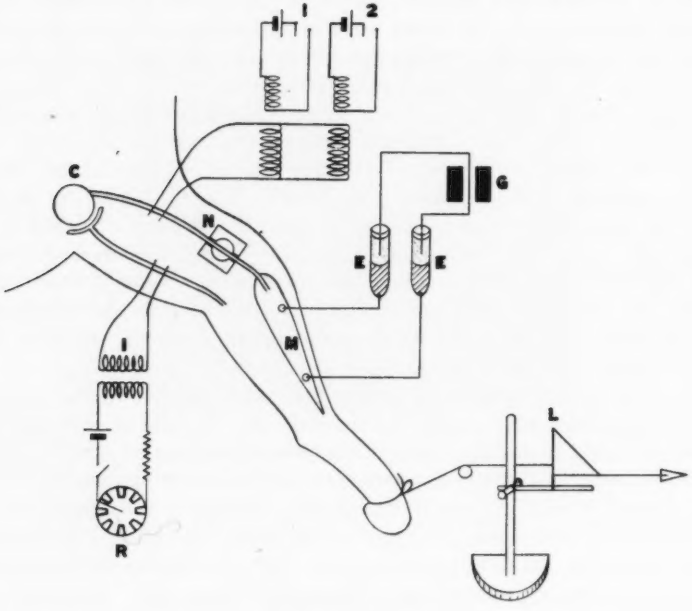

Fig. 2 Diagram of arrangement of preparation and apparatus. *I*, inductorium. *R*, rotary interrupter for tetanizing stimuli to afferent nerve. *C*, spinal center. *N*, narcotizing cup on motor nerve. *M*, tibialis anticus muscle. *1*, *2*, keys of Lucas pendulum for stimuli to motor nerve. *E*, *E*, agar electrodes. *G*, string galvanometer. *L*, recording muscle lever.

applied at 2.5σ disappeared, thus showing total blocking of the second impulse of the pair, the pendulum was reset to give a stimulus interval of 3σ and a new series was taken, to determine the time at which the second impulse set up at this stage of recovery should be extinguished by the narcotic.

When the block became complete, as shown by the failure of even the first stimulus to evoke contraction, the alcohol was removed and replaced

with Ringer's solution. Records showing the return of conduction were made as the effect of the alcohol wore off. The block was thus established and removed three or four times in each complete experiment.

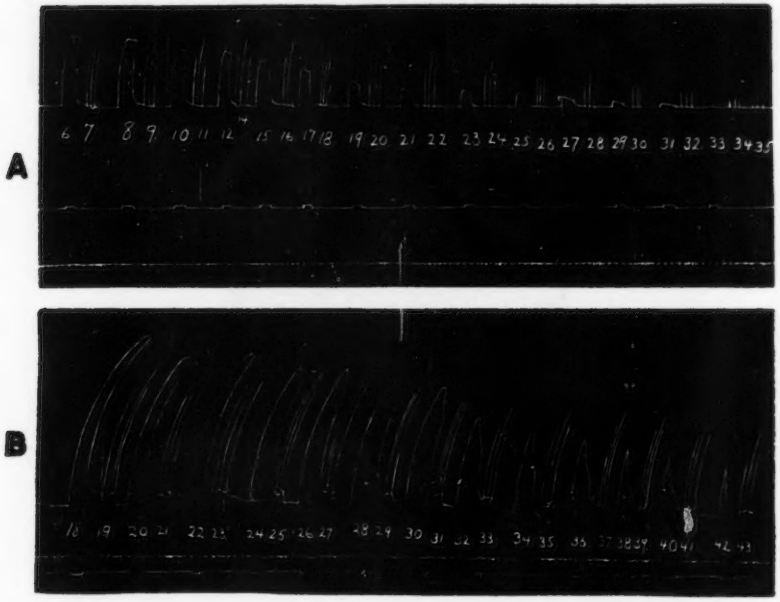

Fig. 3. Progressive narcotization of motor nerve in two preparations without spinal transection. Reflex stimulation with moving drum alternating with motor nerve controls on stationary drum. Each control series consists of single, double and single stimulation in succession; all stimuli maximal; summated contraction measures the passage of subnormal impulses through the block.

A, February 9, 1927; stimulus-interval in controls, 2.5σ. Alcohol applied between records 7 and 8. Total block 9½ minutes later, just after record 35.

B, April 8, 1927; stimulus-interval in controls, 2.5σ. Alcohol applied about half a minute before record 18. Record 43, with summation still present, 12 minutes later.

Below each record a time marker shows seconds when the drum is moving; a signal magnet shows duration of reflex stimulation.

RESULTS. As in the case of the crossed extension reflex, so with the flexion reflex, most of the impulses of central origin appeared usually to be more readily blocked than the full-sized impulses set up by direct stimulation of the rested motor nerve. Evidence to this effect appeared in all of the eleven experiments in which observations on the flexion reflex were made. Typical series of observations are recorded in figures 3, 4A and 5A.

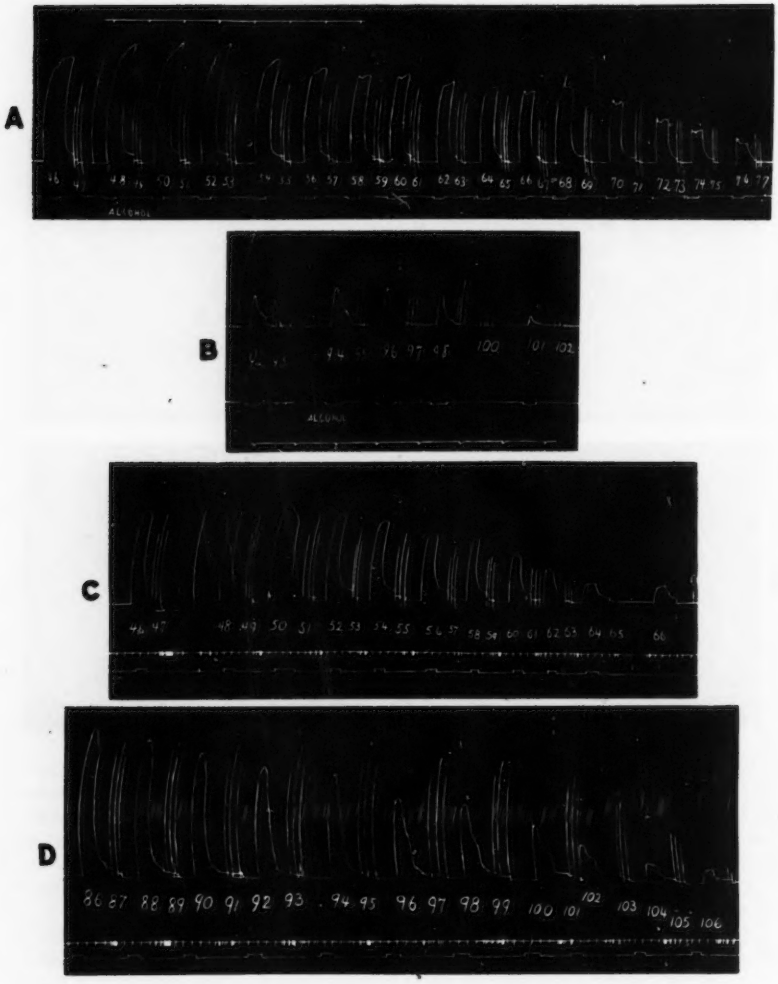

Fig. 4. Records from two preparations both before and after spinal transection. Procedure as in figure 3. A and B, January 31, 1927; C and D, February 7, 1927. A and C, before spinal transection; B and D, after. Records begun 8 minutes and 20 minutes after transection in B and D respectively. Time of application of alcohol shown on records A and B; applied after no. 47 in C, and 2½ minutes before the first record in D.

Stimulus-interval in controls, 2.5σ except after no. 69 in A and after no. 61 in C, where it was increased to 3.7σ, with resulting return of summation in A.

In A and B time is shown in 5 second intervals; in C and D in 1 second intervals. Duration of reflex stimulation shown by downward excursion of signal in A and B, by upward excursion in C and D.

The drum was stationary during the control tests in which stimuli were applied to the motor nerve. It was moving slowly during the course of reflex stimulation. It will be seen that before the application of the narcotic, reflex contraction was typically well maintained; also there was a large

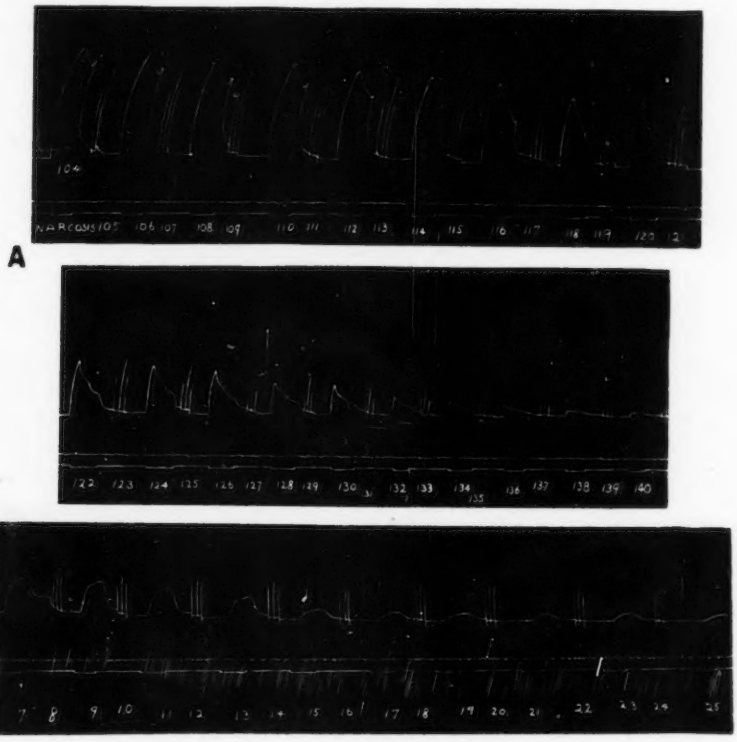

Fig. 5. Comparison of flexion and crossed extension reflexes. No spinal transection. May 19, 1927.

A, flexion reflex. Alcohol applied 50 seconds before first record. Total block 1 minute after last record, 15 minutes after alcohol applied.

B, crossed extension reflex in same animal. Alcohol applied about 1 minute before first record. Stimulus-interval in all, 2.5σ.

measure of summated contraction resulting from a second motor-nerve stimulus applied at 2.5σ after the first. As the narcotic begins to act, the reflex contraction decreases slightly in amount and becomes conspicuously less well maintained. At the same time there is very little decrease in the height of contraction resulting from the single stimulus applied to the

motor nerve, indicating that in only very few fibers are full-sized impulses blocked; but the amount of summated contraction has become considerably less, indicating that in a much larger proportion of the fibers the subnormal impulses set up after an interval of 2.5σ have been extinguished. Before the summated contraction has entirely disappeared, and therefore while some of the fibers are still able to conduct these subnormal impulses, the reflex contraction has become characterized by a sharp initial contraction followed by a very rapid decline. This effect became rapidly more marked, in most experiments, as the narcosis deepened. The inference seems to be that the flexion reflex begins with a considerable volley of full-sized impulses.

The rapid subsequent decline of contraction seems to indicate that the maintenance of the reflex after the initial volley depends to a considerable extent on subnormal impulses, that is, impulses following each other with fairly high frequency in the motor nerve. In our preliminary report (1927) we drew the inference that the dominant frequency was not less than 400 per second, after the first volley.

In one preparation we made a comparison of the crossed extension and flexion reflexes. First we repeated the experiment on the crossed extension reflex, as was done by Forbes and Olmsted, but with the more complete control employed in the present investigation. After this experiment we repeated our usual observations on the flexion reflex in the same preparation. A comparison of the effect of the alcohol block in the two reflexes is illustrated in figure 5. The evidence seems to show clearly that in this preparation the crossed extension reflex depended more largely upon subnormal impulses than did the flexion reflex. In the former the reflex contractions had decreased to a small fraction of their former size when summated contraction still showed that in many fibers subnormal impulses set up at the 2.5σ interval were still able to get through the block. In the case of the flexion reflex, the usual decline of contraction following the initial peak began to appear early in narcosis, even before the summated contractions in the controls had been greatly impaired. Yet the reflex contractions were relatively better maintained, as compared with the responses to single direct stimuli, than was the case with the crossed extension reflex. The inference is that the average frequency of motor-nerve impulses was higher in the extension than in the flexion reflex.

It is noteworthy that our results illustrate the difference between the two reflexes already emphasized by Liddell and Sherrington (1923) and by Cooper and Adrian (1924). The abrupt onset of the flexion reflex, in a previously inactive center, with an almost synchronous volley of impulses, becomes more apparent when the subnormal impulses are blocked by narcosis. This initial volley of full-sized impulses penetrates the narcotic block and causes an initial contraction resembling the neuro-myal twitch.

In the crossed extension reflex with its gradual onset by "recruitment" of increasing numbers of motor neurones, there is no such initial volley.

In several experiments the effect of spinal transection was examined. To this end the spinal cord, at the level of the last rib, was exposed before decerebration. After the experiment had been performed as usual, the cord was transected, and the experiment was repeated. Examples of the results before and after spinal transection are shown in figures 4, 6 and 7.

In four experiments in which the flexion reflex was investigated with the procedure described above, both before and after spinal transection, results of the type illustrated in figure 4 were obtained. In these it will be seen that before the narcotic block was applied, the contraction was better sustained during continuance of the stimulation before spinal transection than after. Before transection the contraction remained nearly at peak height, or even continued to increase, throughout stimulation; after transection a marked decline of contraction began about 1 second after the commencement of stimulation. The effect of partial narcosis was not greatly different in the two cases, but the early decline of contraction after transection was perhaps accentuated by the narcotic block. This would tend to indicate a slightly higher frequency of motor-nerve impulses after transection than before, but on that point the evidence is not clear enough to be at all convincing.

The possibility of confusion due to selective blocking of proprioceptive impulses was controlled by Forbes and Olmsted in the case of the crossed extension reflex. Although the flexion reflex is less liable to this source of confusion, we de-afferented the tibialis anticus muscle, in one of our experiments. For this purpose we cut the dorsal roots of the 6th, 7th and 8th post-thoracic nerves immediately before decerebrating the animal. This rendered it impossible to evoke the flexion reflex by stimulating the popliteal nerve, and led us to use instead the anterior crural.

As a preliminary to this experiment it was desirable to see whether the reflex produced by the anterior crural nerve differed from that evoked through the popliteal. Therefore we made two complete experiments, using both nerves on animals with intact dorsal roots, including observations both before and after spinal transection. In one of these (May 13) stimulating electrodes were secured in contact with both nerves throughout the experiment, and they were used in regular alternation to evoke the reflex.

After these two experiments, we performed a complete experiment in a de-afferented preparation, making observations both before and after spinal transection. Thus in three preparations we recorded the reflex as evoked by the anterior crural nerve. In two of these the reflex, before spinal transection, differed strikingly from that evoked by the popliteal nerve. The crural reflex was characterized by a strong but brief initial contraction

followed by an almost complete relaxation during stimulation, suggesting inhibition, presumably representing an extensor component in the central effect (cf. Forbes, 1921). In the remaining experiment of the three (May 13, dorsal roots intact) the unblocked reflex was greater and better sustained when evoked through the crural than through the popliteal nerve. As the narcotic block became effective, the initial contraction decreased more in the popliteal reflex than in the anterior crural, and was followed by the usual increasingly rapid decline, but in the case of the crural nerve there appeared, after the initial contraction, a sharp decline to a lower level, followed by a slight gradual increase in contraction. The course of events

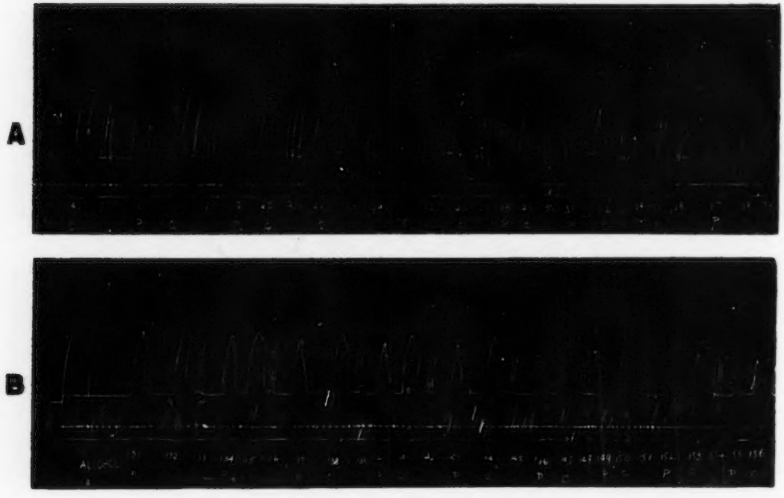

Fig. 6. Comparison of flexion reflex as evoked with popliteal nerve, *P*, and with anterior crural, *C*, during progressive narcosis of motor nerve. May 13, 1927.
A, before spinal transection; B, after.

is shown in figure 6A. These differences appeared consistently through two successive narcotizations and recoveries. The brief initial contraction in the crural reflex, appearing as the block develops, is strikingly suggestive of the brief contraction in the other experiments with the crural nerve. It is a remarkable fact that the similarity was obscured until the block had begun to impair peripheral conduction. This suggests that the persistent contraction in the unblocked reflex was due to nerve impulses of higher frequency than those involved in maintaining contraction in the popliteal reflex. It is hard to harmonize this view with the hypothesis of inhibition, suggested by the other anterior crural preparations. The fact that the

decline after the initial contraction was so marked in the de-afferented preparation suggests that possibly proprioceptive impulses helped to maintain contraction in the unblocked reflex of May 13 (fig. 6A), and that the narcotic acted first on the afferent fibers which conducted them.

After spinal transection in all three experiments with the crural nerve the picture changed strikingly. In the experiment in which both nerves were used (May 13) the popliteal reflex became the greater of the two (fig. 6B). Furthermore when the block became effective the crural reflex no longer declined sharply after the initial twitch, but was even better

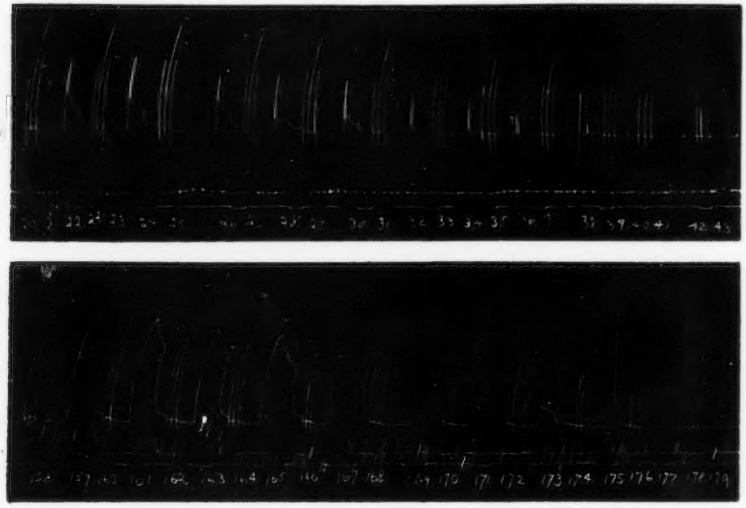

Fig. 7. Flexion reflex in de-afferented preparation; A, before; B, after spinal transection. Reflex evoked through anterior crural nerve. May 20, 1927.

A, alcohol applied 6 minutes before first record; stimulus-interval, 2.5σ.

B, beginning 21 minutes after spinal transection; alcohol applied 5 minutes before first record; stimulu-sinterval, 2.3σ.

sustained than the popliteal reflex. Again the differential effect was consistent throughout narcotization and recovery. Apparently spinal transection caused changes in the temporal sequence of motor-nerve impulses (peroneal) in the flexion reflex, and these changes were different in the case of the popliteal nerve from those in the case of the anterior crural. This matter will be considered presently in connection with the electrical records.

Examples of the records obtained in the de-afferented preparation are shown in figure 7. Except in the points already mentioned, and which

seem to reveal properties peculiar to the anterior crural nerve, the results were essentially the same as those already described. After spinal transection the results differed little from those regularly obtained under like

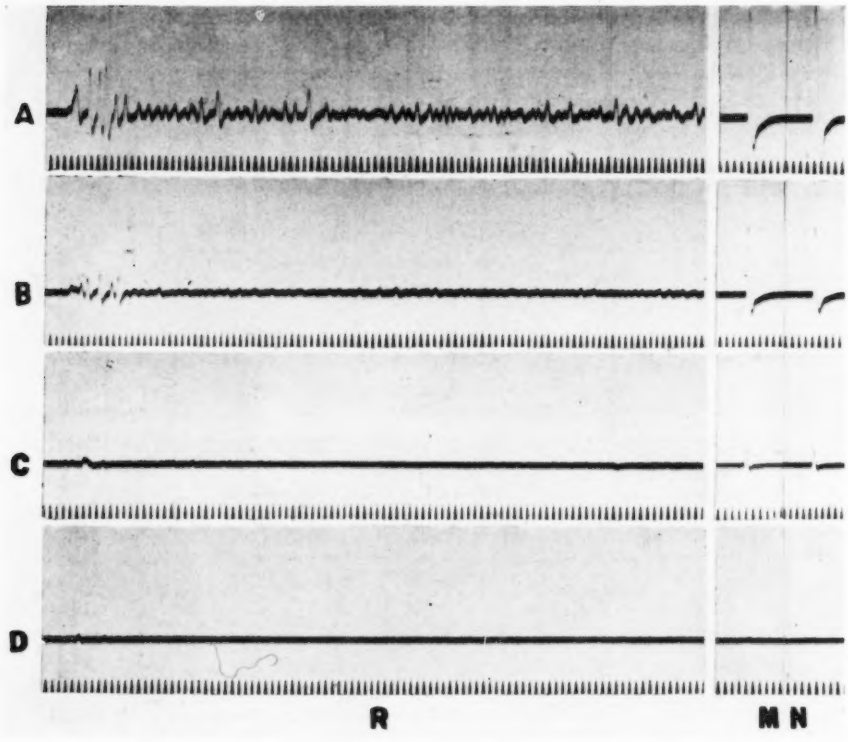

Fig. 8. Electromyograms from the experiment shown in figure 4A. All records taken during the course of a single narcotization, before spinal transection. *R*, reflex responses; *MN*, responses to motor nerve stimuli, first single, then double. The second response on double stimulation appears merely as a delayed return of the string from the peak of the excursion in the last record of A, made before the block was effective. The electromyograms are identified with the drum records in figure 4A, as follows: A, 48 and 49; B, 66 and 67; C, 76 and 77; D, 2½ minutes later as block was becoming complete.

Time below; 1 d.v. = 0.01 second.

conditions from the preparation with dorsal roots intact, on stimulating the popliteal nerve. This harmonizes with the evidence of Liddell and Olmsted (1929) which indicates that the afferent and motor impulses are extinguished by about the same degree of narcosis.

The action currents recorded from the tibialis anticus muscle supported the inferences drawn from the smoked drum records, with which they were simultaneously made. At certain stages of narcotization the relatively greater blocking of impulses during the continuance of reflex stimulation as compared with the response to the single motor-nerve stimulus, was more apparent in the electromyogram than in the mechanical record. This is illustrated in figure 8, in which are shown samples of the electromyograms corresponding to the drum records in figure 4A. In the electrical records the onset of the unblocked reflex is marked by a series of large excursions which must represent more than a single volley in the motor neurones. The rapid subsidence in the size of the excursions, as stimulation continues, may mean a preponderance of subnormal responses in the individual muscle fibers, due to their high frequency of discharge, or a loss of synchronism as the responses in the different fiber groups get out of phase with each other. The decrease in the reflex response, as the narcotic block takes effect, is most marked in the sustained portion of the reflex, i.e., after the initial group of large excursions. The blocking of the initial volley of the reflex is little or no greater than the blocking of the response to the motor nerve stimulus. In figure 8D, representing a part of the drum record just beyond what is shown in figure 4, a few fibers revealed the initial volley of the reflex by an excursion of about the same size as the response to motor-nerve stimulation at the same stage of narcosis, but after one more smaller excursion the muscle became apparently perfectly quiescent throughout the remainder of the reflex stimulation. Thus it appears that, in the nerve fibers which were not wholly blocked, virtually all subsequent impulses were subnormal in size.

Figure 9 shows the effect of spinal transection on the electromyograms taken during the progressive action of the block; it is taken from the experiment shown in figure 4, C and D. Before transection the picture is essentially the same as in figure 8 (also taken before spinal transection), although the excursions in the reflex are smaller. After transection the initial volley in the reflex is greatly intensified. Also the effect of the block on the continuance of the reflex is slightly greater after transection than before, which suggests that the nerve impulse frequency, after the initial volley, is higher after spinal transection than before. This same series of changes in the electromyogram appeared in another of those experiments in which spinal transection caused the characteristic changes in the mechanical effects of the narcotic block, described above. Again the evidence, though not definite enough to be conclusive, tends to confirm the evidence of the mechanical records. The total absence of action currents after the initial reflex volley in all electromyograms including and after no. 106 of February 7 (see figs. 4 and 9) showed that the small persisting mechanical excursions were due to traction from other muscles, in consequence of imperfect fixation.

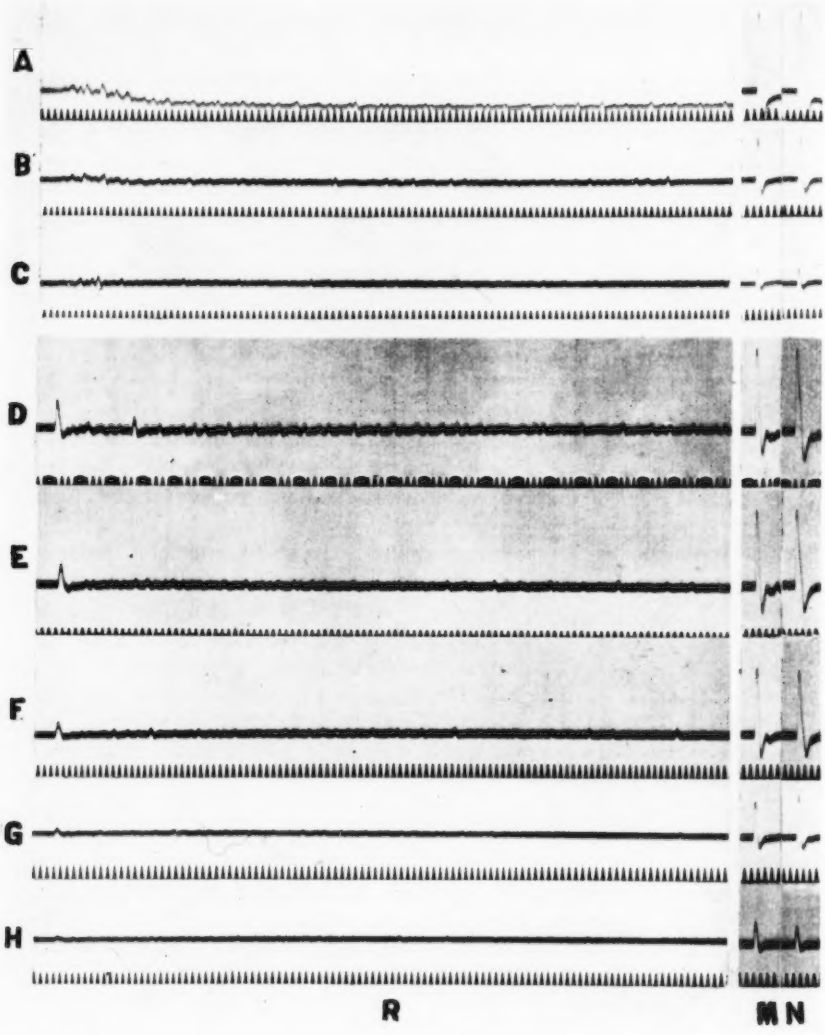

Fig. 9. Electromyograms from the experiment shown in figure 4, C and D. A, B and C correspond to records 46 and 47, 58 and 59, 62 and 63, respectively in figure 4C. (Before transection.)

A is before application of alcohol; B, 6 minutes after application; C, 7 minutes after application. Stimulus-interval in controls, 2.5σ in A and B; 3.7σ in C.

D—H correspond to records 84 and 85, 94 and 95, 98 and 99, 102 and 103, 104 and 105 in figure 4D. (After transection.) Stimulus-interval in all controls, 2.5σ. The second response on double stimulation (last column) can be seen as a delayed descent of the string in all but the last stages of narcosis (C and H).

In figure 10 are shown sample electromyograms from the experiment (May 13) in which both popliteal and anterior crural nerves were used to evoke the reflex. The records are selected from the same parts of the experiment that are illustrated in figure 7, and they show the course of narcosis of the motor nerve both before and after spinal transection. It is interesting to note the correspondence between electrical and mechanical records in this series. Correlated with the mechanically better sustained response to crural than to popliteal stimulation, before spinal transection, we find the amplitude of the electrical excursions also better sustained in the crural reflex. In figure 10A the crural record shows an action current rhythm of about 25 per second appearing after about half a second of stimulation. It does not appear in the popliteal record, nor was it evident in the first record of the crural reflex before alcohol was applied to the nerve. The records in figure 10A were made about two minutes after application of the narcotic, but before its blocking effect was appreciable. The same action current rhythm reappeared in the crural records when the block had been removed and normal conduction was restored.

Before spinal transection, the effect of the narcotic block was to stop the reflex discharge after the first few volleys more completely in the case of popliteal than in the case of crural stimulation. In the crural reflex, the slight secondary increase in contraction following the sharp decline, noted in the mechanical records, appears in the electromyogram as a steady persistence of action currents at a time when, in the popliteal reflex at the same stage of blocking, they have almost disappeared. Thus the records, both mechanical and electrical, suggest a higher frequency of discharge when the reflex is evoked by the popliteal nerve.

After spinal transection, the difference between the popliteal and crural reflexes largely disappears in the electrical as in the mechanical records, although the crural reflex still persists better in the late stages of blocking than the popliteal. This is shown in the last half of figure 10.

In figure 11 are reproduced electromyograms from the first preparation in which the anterior crural nerve was used to evoke the reflex. The drum records from this experiment are not shown, but they resembled closely those in figure 7, from the de-afferent preparation, in which the anterior crural nerve was used; the chief difference is that in this preparation, before spinal transection, the muscle relaxed even more completely after its initial contraction than in the reflexes shown in figure 7A. In figure 11 it will be seen that before transection the reflex began with a group of action currents which lasted about 40σ, after which there was an almost complete absence of measurable excursions; this is correlated with the abrupt relaxation appearing in the drum records. In figure 11 B, taken when the nerve was partially blocked, but still conducting subnormal impulses in some of its fibers, there is no visible trace of action currents after the first

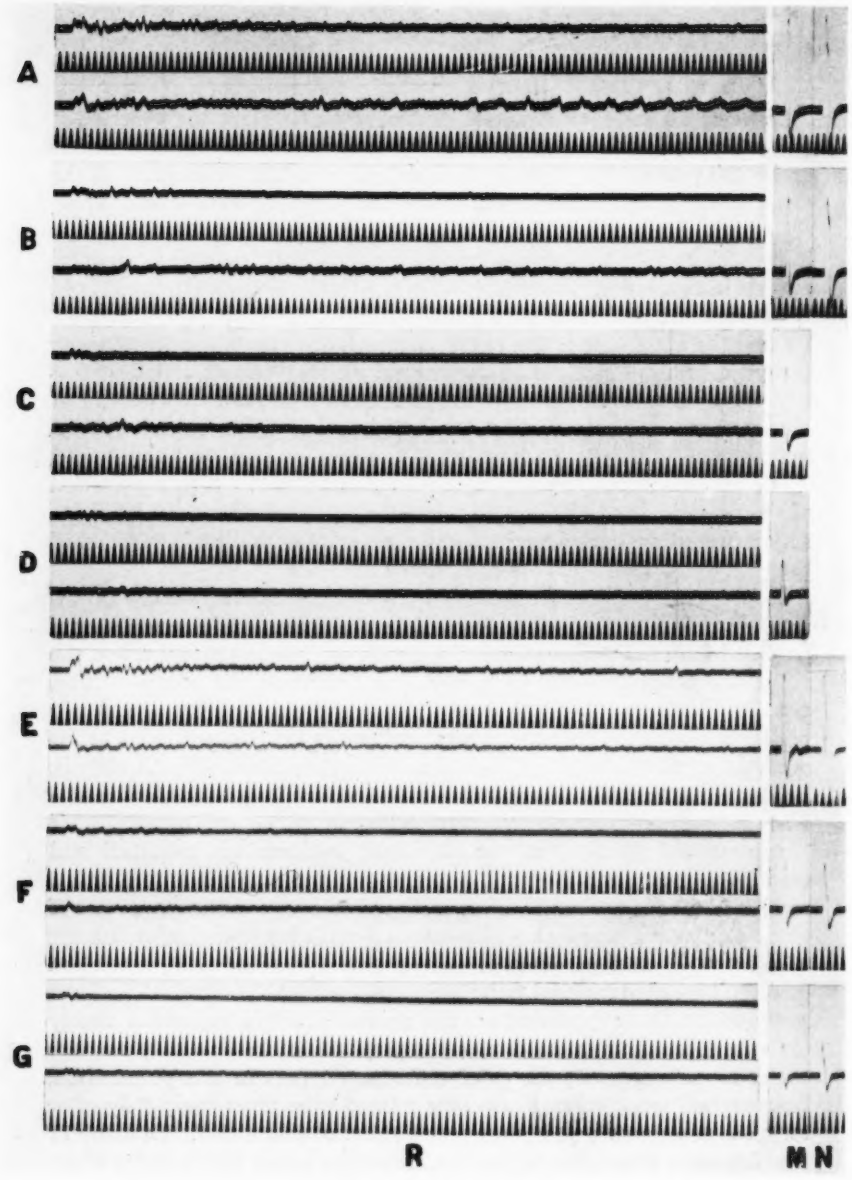

Fig. 10. Electromyograms corresponding to drum records in figure 6. *R*, reflex; *MN*, motor-nerve stimulation. In each reflex group the upper record shows popliteal stimulation; lower record, anterior crural. A-D shows course of one narcotization before spinal transection; E-G, after transection.

Records correspond to those of figure 6 as follows: A, 13–15; B, 25–27; C, 31–33; D, 37–39; E, 131–133; F, 152–154; G, 40 seconds after last record in figure 6, and 10 minutes after application of alcohol. In C and D no electrical records were made of double stimuli to motor nerve.

volley in the reflex. The third record, C, shows the stage when the subnormal impulses were blocked in all the fibers, and full-sized impulses in most of them.

In the last three rows are records, made after spinal transection, at approximately the same three stages of narcosis. In the unblocked reflex

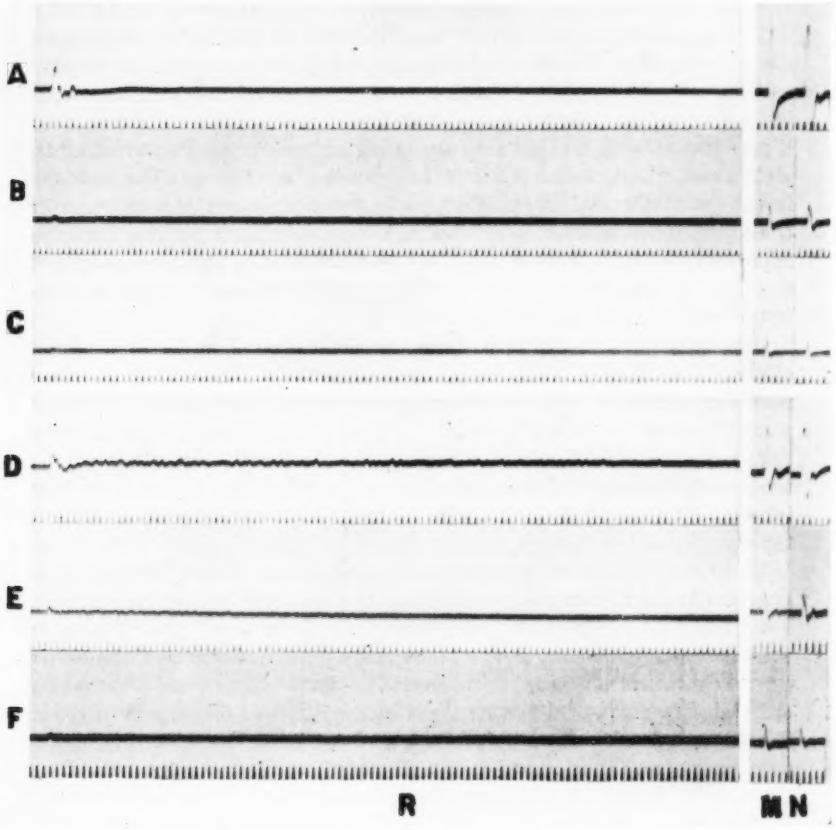

Fig. 11. Electromyograms showing peculiar response to anterior crural reflex stimulation, altered upon spinal transection. May 2, 1927. A-C, before transection; D-F, after.

A, immediately after application of alcohol; C, 15 seconds before complete block. D, 22 minutes after spinal transection, 1 minute after application of alcohol; E, 9½ minutes after application of alcohol in a subsequent narcotization an hour after transection; F, 2 minutes before complete block.

Stimulus-interval, 2.5σ in all but F, in which it is 3.7σ.

(D) action currents persist throughout the continuance of reflex stimulation, and the record differs little from those of the reflex evoked by the popliteal nerve. These continuing action currents persist during the course of narcosis, and even in the last record (F), small excursions are visible for nearly half a second after the initial volley. This record was made when the nerve had just ceased to conduct all impulses set up 2.5σ after the first, but was still able to conduct a few impulses set up at 3.7σ.

It is important to bear in mind that these electromyograms are composite pictures of the activities of thousands of distinct muscle fiber groups, and accordingly must be interpreted on a statistical basis. Absence of visible excursions does not prove complete absence of muscular response; it merely shows that at no time are enough fibers responding together to yield a measurable action potential in the recording circuit. The response in the anterior crural reflex, after the initial contraction, was so small in the experiments of May 2 and May 20, even before narcotization, that the apparently complete quiescence after establishment of the block may not signify a predominantly higher frequency of motor nerve impulses than in the popliteal reflex.

In spite of the uncertainties of statistical interpretation, the differential effect of the narcotic block on the continuance of the reflex, as compared with the initial volley, is great enough in both mechanical and electrical records to warrant the conclusion that a large proportion of the motor nerve impulses, after the first few volleys, are subnormal. In other words, the sustained flexion reflex is mediated by motor impulses whose frequency is so high that most of them are in the relative refractory phase following their predecessors.

We have already mentioned our inference of a frequency of motor nerve impulses in the flexion reflex amounting to about 400 or more per second. Since the publication of our preliminary report, in which this conclusion was set forth, Adrian and Bronk (1929) have published observations which clearly show that this conclusion, as well as that of Forbes and Olmsted on the high frequency of nerve impulses in the extensor reflex, was in error. Adrian and Bronk were able to sever all but one of the active fibers in a motor nerve and then record the responses of muscle fibers innervated by the remaining nerve fiber in both the flexion and crossed extension reflexes. In this way they showed that the individual motor neurone discharges impulses at frequencies between 5 and 50 per second in the case of the flexion reflex, and in the neighborhood of 70 per second in the case of the crossed extension reflex. Their result might conceivably be explained by the blocking of subnormal impulses of a higher frequency at a partial block due to mechanical damage to the remaining active fiber in the operated nerve. But the regularity of the rhythm in the observed response and the persistence of this rhythm after the operation, when the effect of such me-

chanical injury could hardly be expected to remain constant, renders this interpretation extremely improbable. We may therefore accept the conclusion of Adrian and Bronk with confidence.

Evidently there is a fallacy in the reasoning whereby we reached the conclusion that the frequency of motor neurone discharge in these reflexes is several times as high as it has been shown to be by the more direct evidence of Adrian and Bronk. A. V. Hill, on observing our experiment, suggested that an inference as to successive impulses, in a long and rapid series at a given frequency, causing a given degree of subnormality, could not be safely drawn from observations on the blocking of the second of an isolated pair of impulses in a rested nerve. It now appears that Hill's surmise was correct, for Field and Brücke (1926) have demonstrated that on continued stimulation the refractory period becomes greatly prolonged. They found that after repeated stimulation for several minutes, it took several times as long for a nerve to recover sufficiently to respond to a stimulus of a given strength, as after a single response when the nerve had been rested. Through this observation of Field and Brücke we find the reconciliation of our observations with the findings of Adrian and Bronk. Evidently some of the impulses mediating the flexion reflex are blocked by a degree of narcosis which suffices to block impulses set up 2.5σ after a full-sized impulse evoked in a rested nerve. But the succession of impulses in a sustained flexion reflex must produce the same effect as that produced artificially in the experiment of Field and Brücke, and have caused the refractory period to become longer until impulses following their predecessors by as long an interval as 20σ (judging from the frequency found by Adrian and Bronk) are so reduced in size as to be blocked by the narcotic.[1] We see no other way to reconcile these observations.

In order to test this explanation and to determine, if possible, the extent to which the inference of frequency, afforded by the narcotic block method, must be revised on account of the effect of prolonged stimulation, an additional series of control experiments was undertaken. To this end a peripheral nerve-muscle preparation was arranged, consisting of the peroneal nerve and the tibialis anticus muscle, in situ. The nerve was cut at the hip, and stimulating electrodes were applied to the peripheral portion near the cut end. About 2 centimeters distal to the stimulating electrodes was a narcotizing chamber similar to that used in the reflex experiments. Between the narcotizing chamber and the muscle a pair of silver silver chloride leading-off electrodes was applied to the nerve. The leads were about 15 mm. apart. With this spacing the action current appears as a sharply defined diphasic excursion (cf. Forbes, Ray and Griffith,

[1] Liddell and Olmsted (1929), using continued motor nerve stimulation as a control, found evidence that in the crossed extension reflex the frequency of motor nerve impulses was more than 48 per second.

1923). No attempt was made to record the action current of the muscle, but its contraction was recorded as usual with an approximately isotonic lever. The stimulating electrodes were connected with a double-pole double-throw switch whereby the nerve could be stimulated alternately by single or double shocks with the Lucas pendulum or by rhythmically repeated stimuli of any desired frequency. Of this control series the first two experiments were performed on decerebrate cats, the remaining seven on cats anesthetized with urethane or amytal. In the first six the tetanizing stimuli were applied with the rotary interrupter arranged to close the circuit about 50 per cent of the time. In the last three we used instead the electron-tube stimulating device described by Campbell (1929), (cf. Forbes and Rice 1929). This apparatus had two advantages for the present purpose. In the first place, successive stimuli were exactly alike, instead of being alternately in opposite directions, as is the case with the make and break shocks of an induction coil. This prevented the mistaking of alternation in the apparent size of response due to alternation in the strength and polarity of stimuli, for alternation in the number of fibres excited, depending on prolonged refractory phase.[2] The other advantage was that on changing the grid resistance by the simple throwing of a switch, the frequency of the stimuli could be instantly changed to any desired value within the range of the apparatus. In all but the last two experiments the stimulating electrodes, narcotizing chamber and leads were all applied to the nerve in its normal position in the thigh. This technique proved rather awkward, and in the last two experiments we made use of a specially constructed moist chamber mounted close to the knee of the animal, in which the nerve was led successively through the stimulating, narcotizing and leading-off chambers. Warm water circulating through a copper pipe in the bottom of the chamber kept the nerve at a temperature of approximately 30° Centigrade, which was about the same as that obtaining in the leg of the animal in the previous experiments.

The procedure was to set the pendulum to give two stimuli at an interval of 2.5σ, and then to record alternately the maximal twitch and the maximal summated contraction with this stimulus interval, on a stationary drum, then with a slowly moving drum to record the response to the tetanizing stimulation. After a preliminary series a solution of 8 to 11 per cent of alcohol was introduced into the narcotizing chamber, and as in the case of the reflex experiments, records were taken as the block became established, regularly alternating between the pendulum control and the tetanus. With the rotary interrupter the stimulus frequency was usually kept constant throughout the course of a given narcotization at 200 per second;

[2] In our records the electric artefact was appreciable, and since its direction was opposite in make and break shocks, it tended to confuse the question of alternation in the size of response.

sometimes it was set alternately at 200 and 120, and sometimes it was raised to 300 or 400. With the electron-tube stimulating apparatus, the tetanus was usually started with a stimulus frequency of 260 to 300 per second, and then after two or three seconds this was abruptly shifted to about 120 per second. In several observations in the last series three frequencies were used, the last being about 70 per second.

The quantitative interpretation of these control experiments was rendered difficult by the fact that the narcotic does not penetrate the nerve trunk in such a manner as to affect all the fibres simultaneously. This is shown by the decline in the size of the contraction in response to a single stimulus before complete disappearance of the summation which results from a second stimulus applied during the relative refractory period of the nerve. This effect appears clearly in many of our control records, and in those of Forbes and Olmsted. Liddell and Olmsted (1929) have questioned this inference, but we are unable to think of any other possible interpretation of the observation. There is usually a clearer differentiation between subnormal and full-sized impulses during the recovery from the block, when the narcotic is being washed out by Ringer solution. This is undoubtedly due to a more even distribution of the narcotic among the fibres of the nerve trunk. It is apparent both in the mechanical and electrical records.

A comparison of the records of contraction with the electrical records taken from the nerve shows that, because of the approximately isotonic recording of contraction, a relatively small percentage of the fibres in the muscle suffices to give nearly complete shortening during a sustained contraction. The electrical excursions are probably more nearly a linear function of the number of active fibres. For this reason the electrical record is the more sensitive indicator for the early changes when only a few fibres are blocked, whereas in the later stages of narcosis, when a small change in the number of fibres makes a large difference in the mechanical effect, and the action currents are too small to be clearly differentiated from the electrical artefact, the drum record becomes the more sensitive indicator.

In general, the control experiments showed that after only a few seconds of continued stimulation, impulses at comparatively large intervals become blocked by a degree of narcosis, which, in the case of an isolated pair of stimuli, only blocks those impulses following their predecessors by a much shorter interval. This is what we should expect in view of the observation of Field and Brücke, and is added evidence that the refractory period of nerve is prolonged by continued stimulation, so that a given degree of subnormality is found at increasingly long intervals following the preceding response.

An example of this was as follows: in one experiment, when the simple twitch had fallen very little in consequence of the block, but the summation

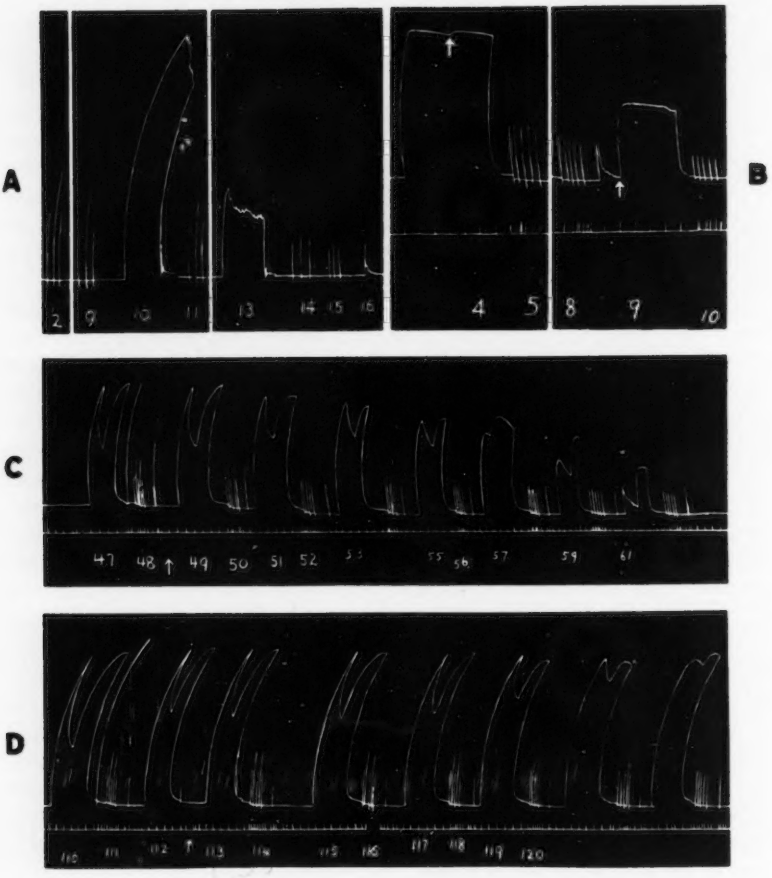

Fig. 12. Control experiments showing alternately pendulum control, and tetanizing stimulation with various frequencies,—all applied to motor nerve. Alcohol block applied as in reflex experiments. All double stimuli at 2.5 σ interval.

A, May 24, 1928. In the pendulum controls, the double stimuli were sandwiched in between two separate single stimuli for comparison. First record shown (no. 2), 3 minutes after application of alcohol. Nos. 10, 13 and 16 show tetanization with 100 stimuli per second (counting make and break shocks), 10, 11 and 13 minutes respectively after application of alcohol.

B, December 17, 1928. Pendulum controls applied in groups of 5, alternately single and double. Tetanizing with electron-tube device, beginning with frequency of 260 per second, changing at arrow to 125 per second. No. 9, showing rapid decline of contraction under high frequency, 4½ minutes after application of alcohol. Time below in seconds.

C and D, December 17, 1928. From other side of the same animal as B. Records showing rapid decline of contraction with high frequency stimuli before narcosis, less rapid decline during early narcosis and (in C) rapid decline reappearing in later narcosis. In C, high frequency 300 per second changed abruptly to 130; in D, high frequency 270 per second changed to 125.

Alcohol (8 per cent) applied after no. 48 in C, after no. 112 in D. Full excursion of lever on stationary drum is shown after the first record in each. Time below in seconds. Drum stationary during pendulum controls. Duration of series shown, 13 minutes in C; 19 minutes in D.

caused by a second stimulus at an interval of 2.5σ had decreased to about $\frac{1}{3}$ of its initial value, the contraction in response to tetanizing stimuli at 200 per second (interval 5σ) showed a rapid decline during the first two seconds of stimulation. A few minutes later, just before summation had entirely disappeared in the pendulum control with the 2.5σ interval, an almost complete Wedensky effect appeared on tetanic stimulation, the contraction rising only to the same height as that evoked by a single pair of summated stimuli and falling within less than a second to nothing. Evidently in most of the fibres the block rapidly became effective to impulses following their predecessors by 5σ, whereas in the rested nerve a considerable number of fibres were still able to transmit impulses set up 2.5σ after the first volley. Examples of this effect are shown in figure 12, A and B. In some experiments the electrical records afforded supporting evidence, in that when the block was beginning to be effective the action currents in the first tenth of a second of tetanization suffered a more rapid decline than they did before the block was applied (cf. Forbes and Rice, 1929), thereby showing that in some of the fibres the impulses were rapidly becoming blocked as stimulation progressed.

The experiments with the electron-tube stimulator were particularly satisfactory in demonstrating the rapid failure of impulses at about 260 per second to pass the block at a stage of narcosis in which impulses at 120 per second were still able to get through in a considerable number of fibres and produce a moderate contraction. The sudden shift of stimulus frequency facilitated these observations. Apparently impulses at a frequency of 120 per second continue to be nearly full-sized even after several seconds of continued activity.

In the course of these observations with the electron-tube stimulator, a new and interesting effect appeared. Before the block was applied, when stimulation was begun with a frequency of about 300 per second, contraction rose rapidly to a much greater height than that of the single twitch or even the summated contraction evoked by two stimuli. But within much less than a second, it rapidly declined again, falling off in about two seconds to a value little greater than the single twitch. When the stimulus frequency was abruptly changed to about 130 per second, the contraction abruptly increased again and rose rapidly to the maximal height. This is seen in the first record of figure 12C. When the alcohol block was applied, early in narcosis and before summation with the two pendulum stimuli had greatly decreased, the rate of decline of contraction during the 300 per second tetanus became progressively less; in some cases the contraction became nearly as well sustained with the stimuli at 300 per second as at the lower frequency. This condition is shown in the middle records of figure 12C, and the last of figure 12D. Later, when the block had just abolished the summation of contraction due to the second of the pair of pendulum

stimuli at a 2.5σ interval, the tetanic contraction again showed a rapid decline beginning immediately after its onset. This appears in the last records of figure 12C.

The rapid decline of contraction in the later phases of narcosis is readily identified with that in figure 12, A and B, depending on the blocking of impulses as they become increasingly subnormal, due to the effect of continued stimulation. The apparently opposite change during the early stage of narcosis is a more puzzling problem. It is evident that with high frequency stimulation, the narcotic has done something to facilitate sustained contraction of the muscle. The most probable explanation is that the rapid decline at high frequency, before the block is applied, is a fatigue effect at the neuro-muscular junction, causing a rapidly increasing number of muscle fibres to fail to respond to the motor nerve impulses. The condition of the neuro-muscular junctions at which this failure occurs may be identified with that producing the classical Wedensky effect. The immediate increase of contractile response, when the frequency of the nerve impulses is lowered, is due to the fact that at the lower frequency the impulses are nearly full-sized and are thus able to excite the muscle fibres at the junctions which failed to conduct at the higher frequency.

The effect of the narcotic block in protecting the neuro-muscular junctions from this fatigue effect can probably be explained only as due to the blocking of alternate impulses in many of the fibres. In our initial control experiments, with a Lucas pendulum delivering three stimuli, we failed to get evidence of any such alternation. When the block sufficed to stop a second impulse, it stopped a third delivered at the same interval. On the other hand, Kato (1929) obtained a condition in which at 15°C., with repeated stimuli at 3σ intervals, the narcotic blocked alternate impulses; the second was extinguished, but the third got through. Earlier in the paper we have mentioned Kato's analysis of the conditions of time intervals and narcotic block, upon which depend the question whether a complete Wedensky effect, i.e., blocking of all impulses after the first, or transmission of alternate impulses, takes place. Probably in most of our experiments the prolongation of refractory period on continued stimulation rapidly established the conditions which Kato found necessary to enable the block to stop alternate impulses, viz., the arrival of every other impulse at the narcotized region during the absolute refractory period in that region. If this is the case, it is easy to see why the contraction became better sustained as the block developed, for the blocking of alternate impulses cuts down the frequency with which they arrive at the neuro-muscular junction to one-half, and therefore establishes a condition similar to that obtained by cutting down the frequency of the stimuli. As alternation became established in an increasing number of fibres, the contractions became better and better sustained, so that there was less room for the recovery of con-

traction when the stimulus frequency was reduced. Finally when the narcosis became deep enough to block all of the impulses in a majority of the fibres, the third stage appeared, viz., that of rapid decline from the initial contraction, under the influence of the block.

A few of the electrical records showed evidence of alternation, but in most cases the number of fibres responding to each stimulus was presumably so nearly equal that alternation could not be detected in this way.

In view of the many fibres, unequally affected by the narcotic, which make up the nerve-muscle preparation, it is impossible with the evidence at our disposal to arrive by the alcohol block method at a quantitative measure of the actual frequency of motor nerve impulses involved in any reflex. It is clear, however, that the frequency in both the flexion and crossed extension reflexes is high enough to render many of the impulses subnormal. It is also clear that the actual frequency is much lower than was inferred from the pendulum control, since continued activity prolongs the refractory period and renders impulses of relatively low frequency subnormal.

SUMMARY

1. The alcohol block method of differentiating between full-sized nerve impulses and those which are subnormal because they occur in the relative refractory phase following their predecessors, has been applied to the analysis of the flexion reflex, as it was in a previous research to the crossed extension reflexes. This was done by subjecting a portion of the motor nerve to gradually deepening narcosis with an alcohol solution.

2. Records were taken of contractions and action currents in a flexor muscle in response to reflex excitation and to single and paired stimuli applied to the motor nerve, both before the alcohol was applied and as the narcotic block became effective.

3. The evidence indicates that the flexion reflex begins with a volley of full-sized impulses. Its continuance is mediated mostly by subnormal impulses, but they are not so easily blocked, and therefore not so far subnormal, as in the case of the crossed extension reflex.

4. The immediate effect of spinal transection on the flexion reflex is usually to accentuate the initial volley and to hasten the subsidence of the reflex discharge during continued stimulation. The evidence suggests that the nerve-impulse frequency may be somewhat higher after spinal transection than before.

5. The effect of the block on the reflex is apparently not due to narcosis of proprioceptive afferent fibres.

6. Interesting differences were observed in the flexion reflex, depending on whether it was evoked by the anterior crural or the popliteal nerve.

7. The motor nerve impulse frequency estimated in a previous research on the crossed extension reflex, and the frequency estimated in the pre-

liminary report of the present research, were much too high, since they were based on a comparison of the blocking effect in the reflex with that observed in a test on the second of two impulses set up directly in the rested motor nerve. Because of the recently discovered prolongation of the refractory period on continued stimulation, these estimates must be revised.

8. This was confirmed in control experiments with continued stimulation of various frequencies, applied to the motor nerve.

9. The control experiments also showed evidence of an early appearance of alternation of response on continued stimulation of the motor nerve with frequencies of about 300 per second.

Miss Rice performed a large and important part of the experiments here recorded. Her untimely death on April 1, 1929, prevented her participation in the final preparation of the work for publication.

We wish to acknowledge the assistance of Dr. Hallowell Davis in several of the control experiments, and to thank Miss Elizabeth F. Lambert for assistance in preparing the paper for publication.

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HORMONES OF THE ANTERIOR HYPOPHYSIS[1]

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Received for publication May 12, 1931

I. METHOD OF ASSAY OF THE HYPOPHYSEAL GROWTH HORMONE WITH THE RAT. The original demonstration in this laboratory of the growth-promoting principle of the anterior hypophysis rested on the response of rats to chronic parenteral administration of anterior hypophyseal substance. Injection was started soon after weaning. Gigantism resulted after some months of daily administration. In the early period of treatment the growth of the injected animals did not far outstrip that of the controls, but the discrepancy in growth of the two groups became marked later in life. In order to elucidate this point something must be said regarding normal growth. The normal growth curve of the female rat (see chart 1) can be schematically described as one characterized by a steep ascent, a turn or "knuckle" and finally, a very slow ascent, practically a "plateau." The change in growth-rate, or knuckle itself, occupies a considerable period. It is usually noticeable around 90 days and may be said to stretch to the 100th or 150th day of life, after which time, females do not normally gain more than ten grams in a twenty day period.

The growth curve of animals which have been treated from infancy (see charts 2 and 3) with the growth hormone[2] presents its most striking

[1] Aided by grants from the Committee for Research in Problems of Sex of the National Research Council, and from the Rockefeller Foundation. These funds have been generously augmented by the Board of Research and by the College of Agriculture of this University.

[2] In this series of studies, unless otherwise noted, a standard aqueous alkaline extract of hypophysis was used. The solution is designated as "17A". The preparation of this solution has been described before but will be repeated here for convenience. x grams of fresh bovine anterior lobe are ground in a mortar, with $\frac{x}{2}$ grams clean sand, until reduced to a smooth paste. This mixture is weighed and 2 cc. of distilled water are added for every gram of ground glands. The volume of this mixture is measured and $\frac{3}{8}$ its volume of 0.2 N NaOH is added. As little as 15 minutes of extraction is adequate but the extraction can be allowed to continue in the ice box 12 hours if more convenient. The alkaline extract is neutralized with 0.2 N acetic acid, care being taken to stir constantly during the addition of the acid. An excess of acid is added so that the reaction of phenol red (1 drop of extract in 5 cc. water) is yellow. Alkali is then added cautiously until phenol red indicator shows the first pink tinge.

contrast to the normal curve in the epoch which we are now considering, for in injected animals the knuckle and plateau features are absent, or at any rate singularly postponed in their appearance. There is for a long time a remarkable continuance of the early abnormally rapid growth with only a gradual later moderation of growth rate.

Young animals grow so rapidly under satisfactory conditions that we experienced some difficulty in detecting the acceleration of growth which could be assigned to action of the hormone in early life. This led us to inquire whether in the plateau epoch previously untreated animals might

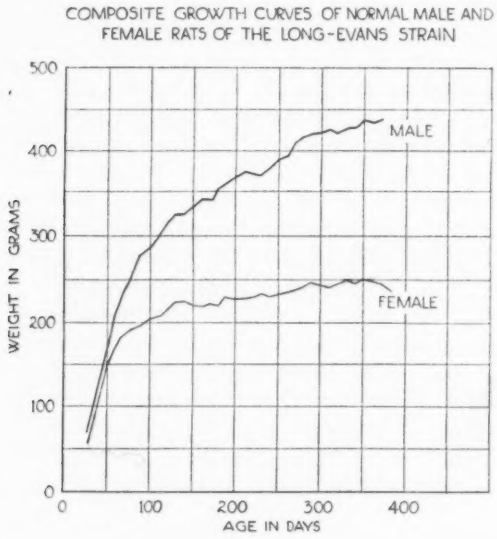


Chart 1

not suddenly resume the very rapid growth characteristic of continuously treated animals at this epoch, and so in a shorter time give clear-cut evidence of the presence of the growth hormone. This was found to be true, and the following procedure was adopted. Normal females with their litter-mate controls are reared and maintained on a satisfactory food mixture. From about the 150th day of life they are weighed at five day intervals to demonstrate that the plateau period had actually supervened. At this time the majority of the females either maintain their weight or

The extract is centrifuged. The supernatant fluid is slightly cloudy and pale pink in color. This extract can be injected into rats without sterilization, but if desired, can be sterilized by passage through a Seitz filter.

gain at a maximum rate of ten grams in twenty days.[3] A minimum of two five day intervals (ten days) is necessary to determine growth stasis.[4] The weighing should, of course, be done at the same time of day. Only

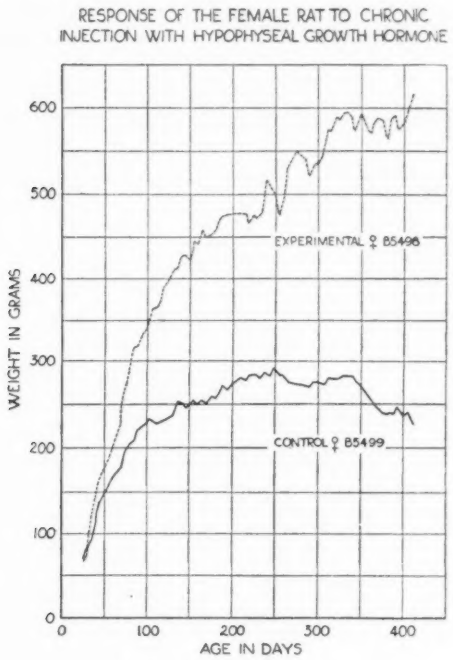


Chart 2

such plateaued individuals are used for growth hormone tests and when a potent solution is conveyed to them the sudden ascent of the growth curve is almost vertical. Chart 4 illustrates the growth test as performed in

[3] It is possible that the handling of the animals involved in weighing and diagnosis is sufficient stimulus to cause some increase in weight. We have analyzed the effect of handling, and also of moving animals from poorly lighted into well lighted quarters to see how much this could affect the weight of a rat five months old or older. Though a gain of fifteen grams occurs occasionally, the gain is usually no more than 4 to 10 grams per 20 day period, averaging 7 grams (based on 100 cases).

[4] More frequent weighing will not shorten the test period necessary for detecting appropriate females. We have submitted ourselves to the tedium of daily weighings but have found distinct disadvantages in this method. An exaggerated picture of growth fluctuation may be given or the weighings for two or three days show stasis when considerable growth still exists, as shown by a following "spurt."

this laboratory. The curves show that solution A contained no growth hormone and solution B was rich in growth hormone. The arrow indicates the time at which injection was begun. When solution A was injected the

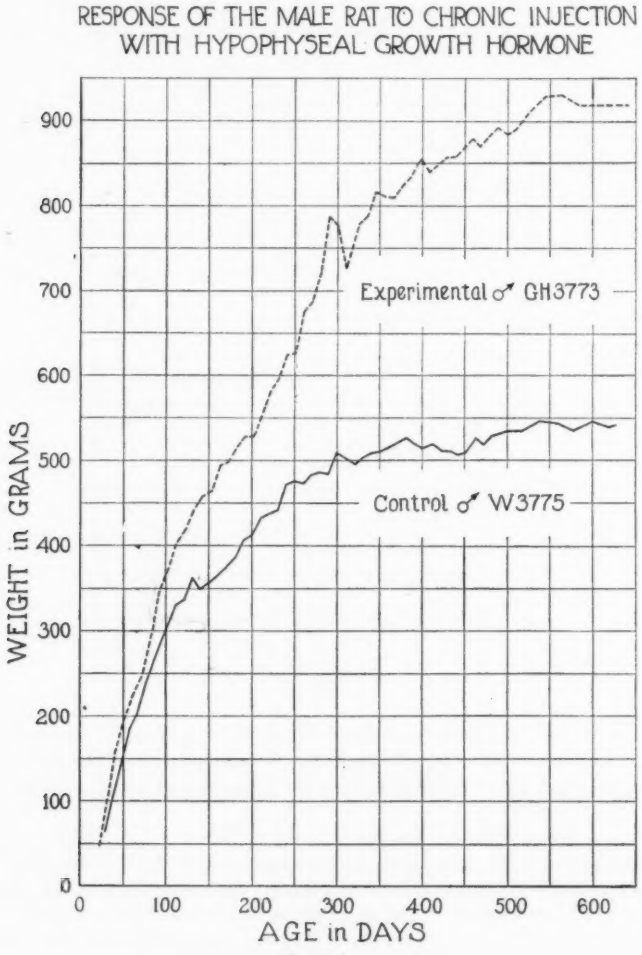


Chart 3

animals maintained the plateau, in the cases in which solution B was injected the plateaued animals suddenly resumed growth. In this way solutions possessing much growth hormone, those possessing some growth

hormone and those not possessing it can be discerned. With a "potent" solution, tested females should exhibit in twenty days an average gain in total body weight of 50 to 55 grams.[5]

Since a considerable amount of fluctuation exists in the responsiveness of animals, it is necessary to use a number for each test. On the basis of a very considerable experience we have concluded that five or six individuals are essential for the test.

In our experience, the same animals can be satisfactorily used for three or four tests if the following conditions are met. The cessation of treat-

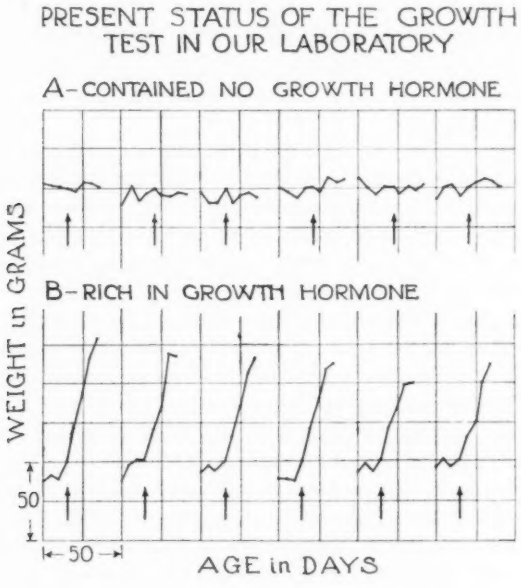


Chart 4

ment with a potent extract not only stops growth but in fact causes for some ten days an actual loss in body weight. A rest of at least a fortnight after cessation of treatment is therefore enjoined. The reëstablishment of the growth plateau must be determined again by weighings (ten to fifteen days). We do not employ animals older than ten or eleven months of of age solely on account of failure to keep maximum vigor and the inevitable greater tendency to infection in later life.

[5] An analysis is included in section II of this paper of the response of large numbers of individuals to a known potent solution.

We should like finally to say a word about the undesirability of males for these tests, although the differing response of males and females to the growth hormone will be made the subject of another section.

The knuckle or the change in growth rate of normal males is not so well marked and occurs very considerably later than is the case with females. While the latter usually exhibit the plateau on or before the fifth month of age, males may not show appreciable slowing at the sixth or seventh month and in all cases the later growth phase in them is characterized by more growth than in females.

These reasons alone are sufficient for the choice of females as test animals. But in addition to this, as the second section will show, males under the influence of the growth hormone are unable to exhibit the same increase in body weight shown by females. Finally females vary less; they are more constant in their response to a given strength of hormone than are males.

II. Sex differences in response of the rat to the hypophseal growth hormone. In the preceding section emphasis was placed on the undesirability of using male rats in tests for the presence of hypophyseal growth hormone. Our conclusion was based, in the first place, on the *characteristics of the normal growth* curve of the male particularly the prolongation of the early period of more rapid growth, and the occurrence of a more rapid growth even in the later phase of the growth cycle than in females; secondly, on the greater *variability of the response* of males during injection of growth hormone; and thirdly, on a surprising *smaller capacity of the males to respond* to acute dosage in the test period (20 days). In this section we propose to discuss in further detail these sex differences in the response of the rat to the growth hormone.

Because of a desire to breed selectively strains sensitive and insensitive to the growth hormone, we have injected very considerable numbers of animals. This gave us the opportunity to observe the differences in responses of a very large number of litter mate brothers and sisters. All animals used in these studies were injected daily intraperitoneally with a fresh potent and standardized extract of the growth hormone.[6] Females were injected at the 150th, and males not until the 180th day of life. The beginning of daily injection of the males was delayed on account of the later attainment of the period of slower growth in males. The group of 907 females used in this study was gaining at an average rate of 3.4 grams in the 20 days, and the 472 males were gaining at the rate of 9.1 grams in the 20 day period preceding injection.

The average growth of the females during the 20 day injection period was 52.20 $\pm$ 0.31 grams. The males treated in the same way gained on the average only 33.25 $\pm$ 0.55 grams. This slighter growth of males is all

[6] See section I of this paper for description of extract 17A.

the more surprising when one considers that the normal growth of males in the "plateau" period exceeds that of females. This difference in the capacity of males and females to respond to growth hormone is graphically illustrated in chart 5. The gains were classed in ten gram groups. As many more females were studied than males the proportion of animals included in each group was plotted rather than actual number of individuals. The contrast in response of males and females is illustrated further in the following short table (table 1). It will be seen that the proportions of males and females gaining set amounts (20, 30, 50 grams) are significantly different.

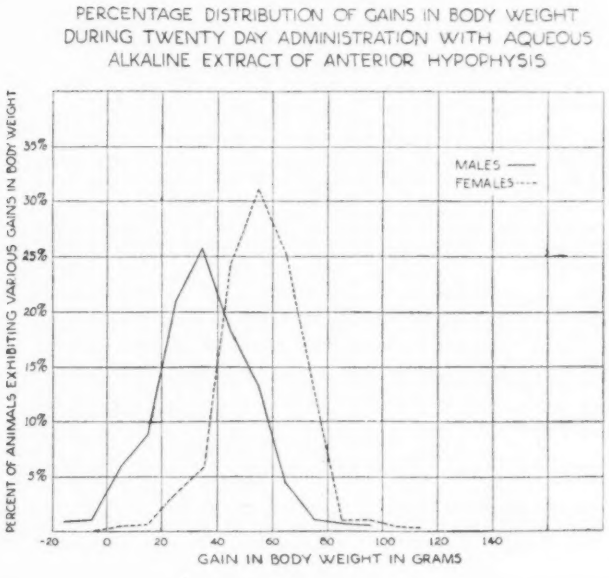


Chart 5

That the response of the male to growth hormone is also more variable than that of the female is illustrated by the following results. The standard deviation of the male from the mean, 33.25 ± 0.55 grams, is 17.75 ± 0.39 grams, the standard deviation of the female from the mean, 52.20 ± 0.31 grams, is 13.65 ± 0.21 grams. This difference between the standard deviation of the males and the females is great enough to be significant $\left(\frac{\text{Diff.}}{\text{P.E. Diff.}} = \frac{4.10}{0.45} = 9\right)$. The curves exhibiting percentage distribution of gains in body weight of males and females show this greater variability of the male (chart 5). In interpreting these curves, attention is drawn

to the following facts: 308 females, or 33.9 per cent, are present in that particular 10 gram class in which the average gain for the whole group falls (50–60 grams); 125 males, or 26.5 per cent, fall in an analogous class (30–40 grams). This is a significant difference $\left(\frac{\text{Diff.}}{\text{P.E. Diff.}} = 4.5\right)$.

TABLE 1

*Illustrations of the difference in response of males and females treated with a standard hypophyseal preparation (17A)**

SEX	NUMBER OF INDIVIDUALS	PER CENT OF TOTAL NUMBER OF INDIVIDUALS	GAIN	DIFFERENCE / P.E. OF DIFFERENCE
			grams	
Females	547	60.2 ±1.1	>50	27
Males	78	16.5 ±1.2	>50	
Females	10	1.10 ±0.23	<20	15
Males	85	18.0 ±1.2	<20	
Females	41	4.51 ±0.46	<30	22
Males	184	39.6 ±1.5	<30	

* The extract, 17A, is described in a footnote on the first page of this paper.

TABLE 2

Dose level at which the standard alkaline extract of the anterior hypophysis (17A) is able to produce maximal growth in the adult female rat

(Any growth approaching or exceeding 50 grams is considered maximal)

LEVEL OF DOSAGE OF 17A	GAIN IN 20 DAY PERIOD	
	Individual results	Average
cc.	*grams*	*grams*
$\frac{1}{16}$ (intraperitoneal)	2, 12, 20, 26, 40, 50	25
$\frac{1}{16}$ (subcutaneous)	10, 12, 18, 20, 30	20
$\frac{1}{8}$ (intraperitoneal)	15, 30, 35, 50, 60, 65	43
$\frac{1}{8}$ (subcutaneous)	10, 40, 40, 45, 45, 50	38
$\frac{1}{4}$ (intraperitoneal)	50, 53, 55, 60, 60, 80	60
$\frac{1}{4}$ (subcutaneous)	45, 55, 60, 70, 70, 95	66
$\frac{1}{2}$ (intraperitoneal)	53, 56, 65, 70, 80, 105	72
$\frac{1}{2}$ (subcutaneous)	40, 70, 70, 75, 85, 90	72

We were interested to find, if possible, an explanation for the marked discrepancies between males and females in their reaction to growth hormone. Since in all cases males were heavier animals than their sisters the possibility existed that a dosage adequate for maximal growth in the fe-

males was not adequate for the larger animals, the males. We have reliably tested the simple conception that we had possibly given doses inadequate with reference to body weight. The growth extract administered is potent in the standard test animals (adult females) in $\frac{1}{5}$ cc. daily parenterally so that 1 cc. daily was greatly in excess of the minimum dose (see table 2). In addition to this a large group of males (over 100 animals) were injected daily with doses of 2 cc. This increased dose produced no change in the growth response and had the previous dose (1 cc.) been near the limit of effectiveness it is inconceivable that doubling the dose would not have produced marked effects.

TABLE 3

Response of males and females of the same body weight to injection of growth hormone (17A)

WEIGHT GROUP	FEMALES		MALES	
	Number of animals in group	Average gain for group	Number of animals in group	Average gain for group
grams		*grams*		*grams*
240	66	54.8	4	64.5
250	37	53.7		
260	28	52.7	6	27.8
270	12	47.8	7	51.5
280	5	48.5	13	41.5
290	5	60.5	15	32.5
300	3	41.1	25	37.8
310	4	54.5	39	33.9
320			33	35.1
330	3	64.5	21	28.3
340	1	34.5	40	37.5
350			41	35.3
360	1	44.5	30	33.8
	Total 165	Average 53.3	Total 274	Average 35.4

The next problem was whether the poorer performance of the males could be attributed merely to greater body weight. If it were true that the difference in response of males and females could be explained merely on the basis of a difference in body weight, then heavy females of body weight comparable to that of males should show the same poor response to growth hormone that characterized males. By neglecting age, it is possible to compare the performance of males and females of the same weight. Such a comparison is shown in table 3. The light males and heavy females were chosen from 1548[7] injected animals. All animals were included whose weights fell within the same weight range (240–360

[7] This included all the 5 to 6 month animals, 1379, as well as 169 older animals.

grams). It can be seen readily that the heavy females cited here responded to the hypophyseal extracts with far greater growth than that which characterized the males of the same body weight.

Further, if it were true that the poor response of males compared with females could be explained merely on the basis of the males' greater body weight, the same relation between gain and body weight should exhibit itself in the gain of animals of a given sex but of different body weights. In the previous section, the growth response of the unusually heavy female was compared with the response of males of the same weight,

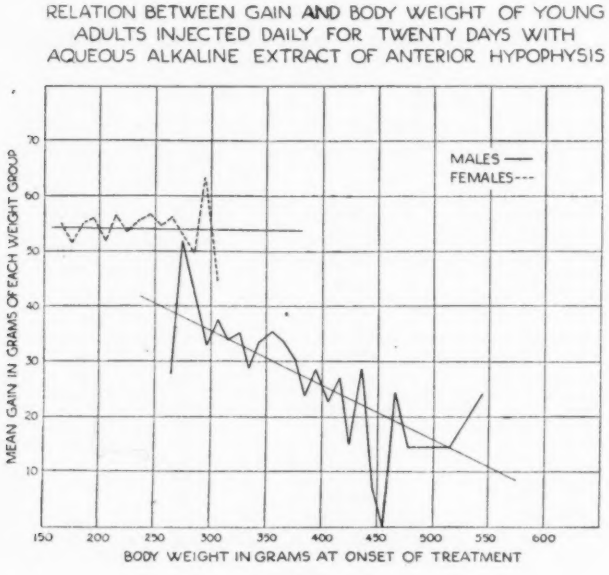


Chart 6

and it was shown that the growth of the females far exceeded that of the males. In fact the heavier females cited in table 3 showed the same responsive capacity, an average growth of 53.3 grams in 20 days, that characterized the whole group of females, mean 52.2 grams. This would indicate that the assumption of a relation between gain and body weight was unfounded. The relation between gain and body weight of the female was therefore analyzed carefully. In the first place the growth response of the heavy and light females within the 5 to 6 month age group was compared. It was found that 68.6 per cent of the lighter females weighing 140 to 210 grams inclusive, gained more than 50 grams and that 70.5 per

TABLE 4

Relation between gain and initial body weight during injection of growth hormone (17A)

WEIGHT GROUP	FEMALES		MALES	
	Gain in 20 days	Number in group	Gain in 20 days	Number in group
grams	*grams*		*grams*	
140	69.5	2		
150	59.5	2		
160	55.1	16		
170	51.2	27		
180	55.1	55		
190	55.6	86		
200	51.9	110		
210	56.5	131		
220	53.3	108		
230	55.6	70		
240	56.6	54	64.5	4
250	54.5	24		
260	56.4	21	27.8	6
270	52.9	6	51.5	7
280	49.5	2	41.5	13
290	64.5	2	32.5	15
300	44.5	1	37.8	25
310			33.9	39
320			35.1	33
330			28.3	21
340			33.2	38
350			35.3	41
360			33.8	30
370			30.5	28
380			23.6	23
390			28.7	19
400			22.5	15
410			27.0	17
420			14.5	5
430			28.6	12
440			7.3	7
450			0.0	2
460			24.5	1
470				
480			14.5	2
490			14.5	1
500				
510			14.5	2
520				
530				
540			24.5	1

cent of the heavier females, 220 to 300 grams, gained more than 50 grams. A further analysis of the response of females of the whole range of body

weights represented in the 5 to 6 month animals (140–300 grams) showed no correlation between gain and body weight (correlation coefficient = 0.00 ± 0.03). The data given in chart 6 and table 4 show that regardless of body weight, females always gain about 50 to 55 grams.

When similar comparisons were made of the growth of heavy and light males in the 6 to 7 month group, it was found that the males did not appear to behave like females. Males were also divided into two weight groups, heavy and light. In this case the two groups included 240 to 340 gram and 350 to 540 gram animals respectively. In the lighter group 64.7 per cent gained more than the average, 30 grams, while in the heavy group, only 49.5 per cent gained more than 30 grams. This difference in response of the heavy and light animals is significantly large $\left(\frac{\text{Diff.}}{\text{P.E. Diff.}} = 4.7\right)$.

TABLE 5

Effect of age on response to acute (20 day) parenteral administration of anterior hypophyseal growth hormone (17A)

SEX	AGE	NUMBER TESTED	INITIAL WEIGHT	GAIN IN WEIGHT IN 20 DAYS
	months		*grams*	*grams*
Females	5½–6	907	213	52.2
	7½	12	237	55
	8	6	274	60
	8½	17	244	55
	9	6	270	53
	10	12	255	29
	14	6	323	52
	19–20	12	265	49
	25–27	4	260	44.5
Averages for females older than 5–6 months.		75 Total		49.3
Males	6½	472	348	33
	10–18	94	447	30.5

When the relation between gain and initial weight was analyzed for the whole range of body weights, the correlation between gain and body weight was also found to be significant (correlation coefficient = − 0.34 ± 0.02). See chart 6 and table 4. Values were substituted into the equation of the form $y = a + bx$ best fitted to the observations on the relation of gain to body weight. The calculated line for the relation between gain and body weight in the 6 to 7 month male is seen in this chart.

We now feel that this difference between males and females is more apparent than real, and is probably due to the fact that the growth curve of the female rat after 5 to 6 months of age forms almost a true plateau, while on the contrary the curve of growth of the male merely becomes less

steep and does not even approximate a plateau until the male reaches a greater age. This explanation had to be substantiated by injecting animals so old that there could be no doubt that normal growth of the male had subsided to a negligible figure.

A number of groups of female rats of varying ages even including animals as old as 19 to 27 months, were injected. As can be seen in table 5 (also see chart 7) the response of females was not materially reduced in senility. Also 94 males aged between 10 and 18 months were injected with growth hormone. These males gained on the average 30.5 grams in a 20 day injection period which did not greatly differ from the average

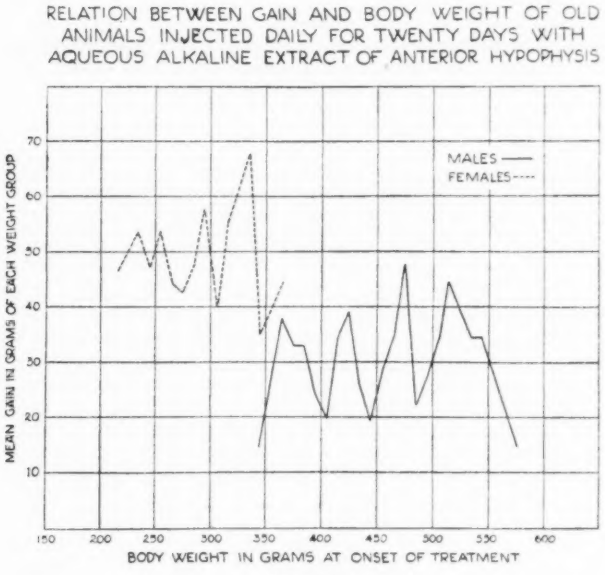


Chart 7

gain of younger males. The relation between body weight and gain, noted in younger males, no longer obtained. As can be seen in chart 7, there was no correlation between gain in body weight among either old males or females. (Correlation coefficient, old males = -0.01 ± 0.07; old females = -0.07 ± 0.08.)

It must be concluded therefore that body weight is not the determining factor in the response to *acute* dosage (20 days) with growth hormone. The results of *chronic* injection of males and females appear to be inconsistent with the findings described above in which injections were made for an acute period. During such a short test period the female gives a much

greater response than the male. However, the largest animals that have resulted from chronic injection of 17A were males; the greatest weight attained by males being 900 grams, the greatest weight of females, being 700 grams. This discrepancy between ultimate size attainable and degree of response during acute injection can perhaps be harmonized. Comparisons were made between the absolute gains of males and females chronically injected from 40 days of age to 220 days and it was found that the males gained absolutely more than the females during every successive 20 day period. However, if one analyzes the difference in response of males and females to chronic injection by comparing net gains of injected animals,

TABLE 6

Comparison of absolute gains and net gains of normal males and females injected chronically with anterior hypophyseal growth hormone

AGE	FEMALES*					MALES				
	Controls		17A injected		Net gain or excess gain of injected over control animals	Controls		17A injected		Net gain or excess gain of injected over control animals
	Weight at onset of each 20 day period	Gain during 20 day period	Weight at onset of each 20 day period	Gain during 20 day period		Weight at onset of each 20 day period	Gain during 20 day period	Weight at onset of each 20 day period	Gain during 20 day period	
days	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*
40	109.8	57.8	108.5	85.1	27.3	120.2	86.6	125.2	90.3	3.7
60	167.6	24.6	193.6	43.8	19.2	206.8	48.6	215.5	54.6	6.0
80	192.2	13.8	237.4	43.8	30.0	255.4	39.4	270.1	47.5	8.1
100	206.0	8.8	281.2	33.6	24.8	294.8	20.8	317.6	60.7	39.9
120	214.8	10.4	314.8	24.4	14.0	315.6	10.8	378.3	25.3	14.5
140	225.2	−5.2	339.2	15.2	20.4	326.4	15.0	403.6	34.7	19.7
160	220.0	−1.6	354.4	22.8	24.4	341.4	11.2	438.3	21.3	10.1
180	218.4	8.0	377.2	12.4	4.4	352.6	14.6	459.6	11.4	−3.2
200	226.4	5.6	389.6	7.6	2.0	367.2	4.0	471.0	14.1	10.1
220	232.0	0.8	397.2	13.2	12.4	371.2	6.0	485.1	31.9	25.9
240	232.8		410.4			377.2		517.0		
Total gains..		123.0		301.9	**178.9**		257.0		391.8	**134.8**

* The number of animals in each of the four classes is six.

i.e., the amount by which gain of 17A injected males and females is greater than that of their normal controls, then one finds that the net gain of the 17A injected female is always greater than the net gain of the 17A injected male. This means that even though the chronically injected female is always smaller than the male chronically injected for the same period, nevertheless the female is the more responsive animal to 17A injection, during chronic injection just as it is during acute injection (table 6). In the series cited in this table, the differences between responses of chronically injected males and females is not so great as it has been found to be in an acute injection period.

The size of the male has not explained its distinctive behavior. The rôle of the gonads themselves should be considered in an attempt of this sort to study differences in the response of males and females. A separate section deals with the reactions of gonadectomized male and female rats to growth hormone. However one table should be inserted at this juncture (table 7) for comparison with table 6, as it appears that the conception of a growth accelerating influence in the testis, or the growth inhibiting power of the ovary can not be introduced to explain the sex differences in reaction to growth hormone. In table 7 it can be seen that the net gain—the amount by which the gain of the injected animal exceeds the gain of its control—

TABLE 7

Comparison of absolute gains and net gains of gonadectomized males and females injected chronically with anterior hypophyseal growth hormone

AGE	GONADECTOMIZED FEMALES*					GONADECTOMIZED MALES				
	Controls		17A injected		Net gain or excess gain of injected over control animals	Control		17A injected		Net gain or excess gain of injected over control animals
	Weight at onset of each 20 day period	Gain during 20 day period	Weight at onset of each 20 day period	Gain during 20 day period		Weight at onset of each 20 day period	Gain during 20 day period	Weight at onset of each 20 day period	Gain during 20 day period	
days	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*
40	104.1	70.4	93.8	81.5	11.1	112.8	53.2	118.0	77.8	24.6
60	174.5	22.1	175.3	70.5	48.4	166.0	60.0	195.8	95.2	35.2
80	196.6	14.9	245.8	34.0	19.1	226.0	29.1	291.0	45.8	16.7
100	211.5	13.1	279.8	38.2	25.1	255.1	11.7	336.8	26.0	14.3
120	224.6	13.7	318.0	37.6	23.9	266.8	32.6	362.8	33.6	1.0
140	238.3	5.7	355.6	22.0	16.3	299.4	13.6	396.4	27.6	14.0
160	244.0	0.3	377.6	42.0	41.7	313.0	10.7	424.0	26.8	16.1
180	244.3	10.3	419.6	23.0	12.7	323.7	6.3	450.8	21.4	15.1
200	254.6	2.0	442.6	20.4	18.4	330.0	8.7	472.2	25.4	16.7
210	256.6		463.0			338.7		497.6		
Total gains..		152.5		369.2	**216.7**		225.9		379.6	**153.7**

* The number of animals in each of the four classes is six.

of the 17A injected gonadectomized female is greater than the net gain of the 17A injected gonadectomized male. The same relation then obtains (see table 6) as in animals with gonads. The results with gonadectomized animals thus indicate basic differences in male and female tissues.

The differences in the response of males and females to growth hormone are clear. Males gain less than females in the twenty day test period. They are less dependable than females in their response, i.e., the variability of males is greater. This study however has not furnished an explanation of the differences between the two sexes. The gains of females (52.2 grams) and of males (33.3 grams) are approximately inversely pro-

portional to their body weights, 213 and 348 grams respectively. This suggested the possibility of an indirect ratio between gain and body weight as causal to the characteristic poorer reaction of the male to growth hormone. If true we assumed such an inverse ratio should apply to the growth of animals of markedly different body weights but of the same sex. No such ratio was found in 5–6 month females, but males of comparable age (6–7 months) did show an inverse relation between gain and body weight. This relation did not apply to older animals. We therefore concluded that the inverse relation observed in younger males had been apparent, not real, and due to the fact that even at 6 to 7 months of age males are still growing at a fast enough rate to obscure the results of these analyses of growth induced by the hypophysis. We had to conclude that body weight was not the determining factor in gain resulting from administration of the growth hormone. So also the presence or absence of either type of gonad was not found to be the cause of the greater female or lesser male response to growth hormone. The male after gonadectomy is still a different animal and is less responsive than the gonadectomized female rat; the tissues of the male and female body, aside from internal secretory gonadal influence, behave differently toward the growth hormone.

III. Effect of gonadectomy on the experimental gigantism produced by anterior hypophyseal extract. Four groups of litter mate brother and sister rats were constituted by placing in each group equal numbers (6) of normal-untreated, gonadectomized-untreated, normal-treated, and gonadectomized-treated animals. The injected animals received intraperitoneally daily, beginning with the 30th day of life, 1 cc. of a standardized extract of the growth hormone designated as 17A. Injections were continued in all cases for eight months. Outstanding gigantism was produced in all the treated groups but the response of the different groups varied in a regular way. The heaviest giants were the injected normal males. The lightest of the giants were the injected normal females. The injected-gonadectomized males and females were intermediate between these two extremes, the injected-gonadectomized males being heavier than the injected-gonadectomized females (chart 8). It can be seen in this chart that the curves of all four types of injected animals are shifted upward but retain the same position in relation to one another, i.e., occur in the same sequence. Although they are in the same sequence, the curves do not remain the same distances apart. The greatest difference between treated animals and their untreated controls, i.e., the greatest net gain of injected animals occurred in the case of gonadectomized females, the least in the case of normal males although the latter treated as described above were the heaviest animals (chart 8 and table 8). In table 8 the summary shows that the sequence of increase in net gain (from 40 to 220 days) is, normal males, 109.3 grams, gonadectomized males 153.7 grams,

normal females, 166.5 grams and gonadectomized females, 216.7 grams. This is the inverse order of greatest final size except in the case of the injected-gonadectomized female which is larger than the injected-normal female.

In table 9 and chart 9 the dimensions of the animals after eight months' treatment are compared. It can be seen that the dimensions of the different groups are related to one another in the same way as are the final body weights. The normal female is the shortest among the four types of uninjected animals, and the normal male is the longest. So also the injected-

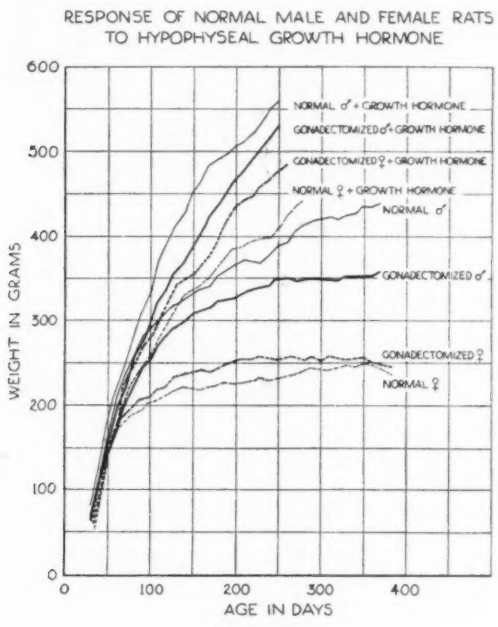


Chart 8

normal male is the longest of the injected animals and the injected-normal female is the shortest. The gonadectomized animals, both controls and injected, are intermediate in length in relation to comparable animals with gonads; the gonadectomized females, injected or uninjected, being longer than similar animals with gonads and the gonadectomized males being smaller than their brothers in which gonads remained. The relation between weight and length attained by the different groups of injected animals was not indentical, however, possibly due to the small number of individuals in each group. The injected-gonadectomized males were heavier than the

TABLE 8

Response of normal and gonadectomized, male and female rats to anterior hypophyseal growth hormone

I. Comparison of body weights

AGE	MALES										FEMALES									
	Normal					Gonadectomized					Normal					Gonadectomized				
	Control*		17A injected		Net gain	Controls		17A injected		Net gain	Control		17A injected		Net gain	Controls		17A injected		Net gain
	Weight†	Gain	Weight	Gain		Weight	Gain	Weight	Gain		Weight	Gain	Weight	Gain		Weight	Gain	Weight	Gain	
days	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*
40	120.6	86.2	125.2	90.3	4.1	112.8	53.2	118.0	77.8	24.6	109.8	57.8	108.5	85.1	27.3	104.1	70.4	93.8	81.5	11.1
60	206.8	48.6	215.5	54.6	6.0	166.0	60.0	195.8	95.2	35.2	167.6	24.6	193.6	43.8	19.2	174.5	22.1	175.3	70.5	48.4
80	255.4	39.4	270.1	47.5	8.1	226.0	29.1	291.0	45.8	16.7	192.2	13.8	237.4	43.8	30.0	196.6	14.9	245.8	34.0	19.1
100	294.8	20.8	317.6	60.7	39.9	255.1	11.7	336.8	26.0	14.3	206.0	8.8	281.2	33.6	24.8	211.5	13.1	279.8	38.2	25.1
120	315.6	10.8	378.3	25.3	14.5	266.8	32.6	362.8	33.6	1.0	214.8	10.4	314.8	24.4	14.0	224.6	13.7	318.0	37.6	23.9
140	326.4	15.0	403.6	34.7	19.7	299.4	13.6	396.4	27.6	14.0	225.2	−5.2	339.2	15.2	20.4	238.3	5.7	355.6	22.0	16.3
160	341.4	11.2	438.3	21.3	10.1	313.0	10.7	424.0	26.8	16.1	220.0	−1.6	354.4	22.8	24.4	244.0	0.3	377.6	42.0	41.7
180	352.6	14.6	459.6	11.4	−3.2	323.7	6.3	450.8	21.4	15.1	218.4	8.0	377.2	12.4	4.4	244.3	10.3	419.6	23.0	12.7
200	367.2	4.0	471.0	14.1	10.1	330.0	8.7	472.2	25.4	16.7	226.4	5.6	389.6	7.6	2.0	254.6	2.0	442.6	20.4	18.4
220	371.2		485.1			338.7		497.6			220.0		397.2			256.6		463.0		
Totals.....		250.6		359.9	**109.3**		225.9		379.6	**153.7**		122.2		288.7	**166.5**		152.5		369.2	**216.7**

* 6 animals in each class. The figures given here are average figures for the class.

† Body weight at onset of 20 day period, gain during 20 days.

injected-gonadectomized females but the average dimensions of these two classes of animals were the same.

IV. THE PERMANENCY OF INCREASED DIMENSIONS AND WEIGHT RESULTING FROM INJECTION OF GROWTH HORMONE. Nothing is more striking than the sudden loss of weight suffered by animals when the daily administration of the growth hormone is stopped (chart 10). The fall in weight is most rapid during the first 10 to 15 days, following which a very gradual loss is evidenced. We endeavored to discover whether such animals did not

TABLE 9

Response of normal and gonadectomized male and female rats to anterior hypophyseal growth hormone

II. Comparison of body length after eight months' treatment

	MALES						FEMALES					
	Control			17A injected			Control			17A injected		
	Nose anus	Anus tail-tip	Total length	Nose anus	Anus tail-tip	Total length	Nose anus	Anus tail-tip	Total length	Nose anus	Anus tail-tip	Total length
	cm.	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*
Normal	23.5	20.1	43.6	26.3	22.2	48.5	19.5	17.2	36.7	25.1	20.3	45.5
	23.1	18.2	41.3	26.4	21.5	47.9	20.6	17.8	38.4	22.9	21.1	44.0
	23.2	19.3	42.5	24.2	20.7	44.9	21.1	17.5	38.6	22.9	20.8	43.7
	24.5	20.5	45.0	26.4	25.5	48.9	21.0	18.2	39.2	22.0	20.4	42.4
				23.2	19.3	42.5	20.2	18.4	38.6			
				26.7	22.9	49.6	20.3	17.2	37.5			
	23.6	19.5	**43.1**	25.5	21.5	**47.1**	20.4	17.7	**37.1**	23.2	20.6	**43.9**
Gonadectomized	22.8	19.2	42.0	26.1	22.2	46.3	19.5	16.2	35.7	22.5	20.9	43.4
	23.6	21.4	45.0	24.3	20.3	44.6	20.8	18.1	38.9	24.3	22.2	46.5
	23.1	19.6	42.7	22.3	22.7	45.0	20.5	18.5	39.0	24.2	22.7	46.9
	21.1	19.7	40.8	24.6	21.1	45.7	19.7	18.6	38.3	23.5	20.2	43.7
	20.1	18.3	38.4				20.5	19.0	39.5	25.4	23.3	48.7
	23.5	20.2	43.7				20.6	17.3	37.9	23.5	22.0	45.5
	22.4	19.7	**42.1**	24.3	21.6	**45.4**	20.2	17.9	**38.2**	23.9	21.9	**45.6**

eventually fall to the skeletal dimension and weight of their normal untreated brothers and sisters but although many cases have been observed for several months and a few for a half-year after cessation of treatment we found they were permanently heavier than their controls (chart 11). In the case of 45 chronically injected female rats, in which decline in weight was followed for an average of 45 days (limits 15–180 days) it was found that an average of 40 grams of the total gain (118 grams) had been lost so the net gain was 78 grams. Not only was there a clear-cut net gain but every single individual of the 45 cases was still markedly heavier than

before injection, and much heavier than females of the same age that had not been injected. In table 10 an experiment is recorded in which animals were injected from the 21st day of life for a period of seven months. After injections were stopped the animals were weighed and measured for seven months longer. These injected animals at 15 months of age were still much heavier animals than their litter mate controls. The post injection loss of weight then was never great enough to reduce them to the weight of their controls. On the other hand any normal gain that occurred subsequent to the post injection loss did not usually bring the weight up to

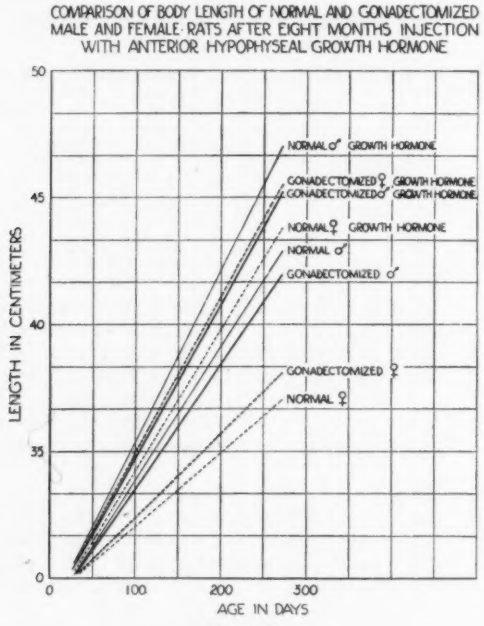


Chart 9

the figure it had reached at the end of the injection period. Similarly the skeletal dimensions attained in experimental gigantism were not diminished in the post injection period. The measurements cited in table 10 illustrate this point. It is also interesting to note that the injected animals even though much longer than their controls, continued to gain in length after injection ceased. Most of them, however, did not gain at as fast a rate as their controls in the post injection period.

V. Treatment of the sporadic dwarf rat with growth hormone. Animal breeders are familiar with the birth of runts, i.e., normally developed, healthy individuals of subnormal weight and stature. These are

encountered rarely in our extensive rat colony. We find that such definitely runted individuals never attain the weight or dimensions of their litter mate brothers and sisters and as time proceeds the discrepancy in the stature of such litter mates gradually increases. We were interested to discover whether the impaired growth exhibited by these animals could be assigned to a decreased capacity to respond to the growth hormone of the anterior hypophysis. Accordingly over a period of a year when such instances of dwarfism were encountered they were segregated and studied. During a 20 day period they were given a standardized alkaline aqueous extract of bovine anterior hypophysis (17A) in 1 cc. doses. Table 11 shows clearly that no significant difference existed in the capacity of runted,

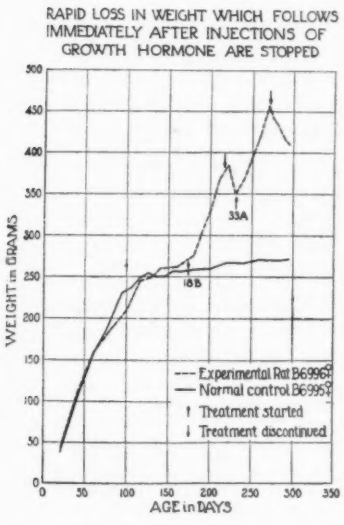


Chart 10

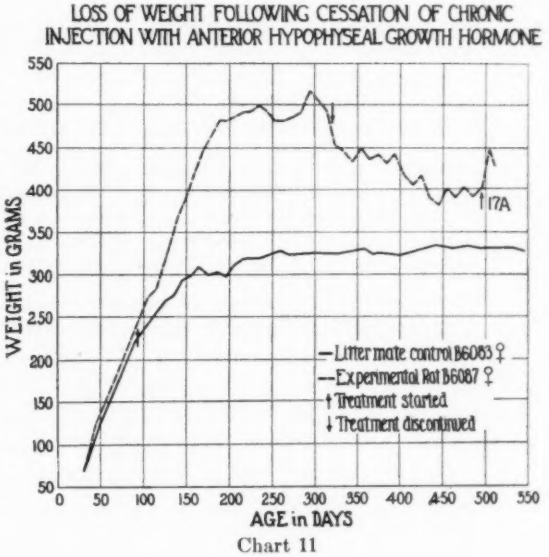

LOSS OF WEIGHT FOLLOWING CESSATION OF CHRONIC INJECTION WITH ANTERIOR HYPOPHYSEAL GROWTH HORMONE
WEIGHT in GRAMS
AGE in DAYS
17A
Litter mate control B6083 ♀
Experimental Rat B6087 ♀
↑ Treatment started
↓ Treatment discontinued

Chart 11

as contrasted with normal, brothers or sisters to respond to the growth hormone.

Since the impairment of these animals was in all instances ante-natal, the efficacy of the growth hormone in increasing their stature is all the more remarkable. The results indicate that some types of congenital dwarfism are clearly amenable to hormone restoration to normal stature.

VI. Effects from hypophyseal implants in senile male rats. The striking effect of hypophyseal implants in accelerating the sexual maturity of rats has naturally led investigators to inquire into the possibility of repairing atrophy and restoring function of the senile sexual apparatus.

TABLE 10

Permanency of dimensions attained as a result of administration of anterior hypophyseal growth hormone

GROUP	DESIGNATION OF ANIMAL	BODY WEIGHT		BODY LENGTH					
				End of injections			After 7 months		
		End of injection period	After 7 months	Nose anus	Anus tail-tip	Total	Nose anus	Anus tail-tip	Total
		gms.	*gms.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*
Normal	B7608 Male	480	584	23.5	20.1	43.6	25.6	20.2	45.8
Injected*	B7609 Male	576	550	26.3	22.2	48.5	26.5	22.6	49.1
Normal	W7786 Male	380	480	23.2	19.3	42.5	25.0	19.6	44.6
Injected	W7787 Male	520	530	26.4	22.5	48.9	25.8	23.4	49.2
Normal	W7597 Male	398	480	24.9	13.0	37.9	25.2		(Tail cut)
Injected	W7598 Male	605	550	26.7	22.9	49.6	26.7	21.7	48.4
Normal	W7532 Female	252	260	20.2	18.4	38.6	20.6	18.7	39.3
Injected	W7533 Female	434	348	22.9	21.2	43.8	23.7	21.6	45.3
Normal	W7469 Female	240	245	20.3	17.2	37.5	20.6	17.6	38.2
Injected	Bh7470 Female	396	332	22.0	20.4	42.4	22.7	20.3	43.0

* Injections 1 cc. daily of a standardized neutralized alkaline extract of anterior hypophysis (17A): injections daily for 7 months from weaning (21st day of life).

TABLE 11

Response of dwarfed rats to anterior hypophyseal growth hormone

SEX	AGE AT ONSET OF INJECTIONS	DESIGNATION OF ANIMAL		INITIAL WEIGHT		GAIN DURING INJECTIONS OF GROWTH HORMONE			
						20 days		45 days	
		Runt	Control	Runt	Control	Runt	Control	Runt	Control
	months			*grams*	*grams*	*grams*	*grams*	*grams*	*grams*
Females	2½	G1525	GH1526	154	200	86	78	170	145
	6	BH9249	BH9248	150	179	60	61	130	113
	8	B7490	B7491	202	232	48	66	100	138
Average				167	203	64	68	133	132
Males	2	BH3315	B3316	74	190	97	102		
	12½	W5706	W5708	319	468	53	17		
	11½	BH5426	GH5427	414	613	22	27		
	6	B8885	B8883	346	465	36	55		
Average				288	434	52	50		

Experience has acquainted us with the real difficulty in deductions as to the functional state of the male sexual system. We often see a singularly unaccountable variation in sex interest displayed by normal males which have been reared, maintained and studied under conditions believed to be ideal and constant. The variation has seldom been stressed by students of sexual physiology but it has induced us to doubt the outcome of studies of sex behavior which do not bear evidence of longer and better controlled study than is usually the case. This must be our excuse for recording here a short re-study of this particular theme. Among a group of a dozen or more male rats still found to be in health and fair vigor at the twenty-first month of life, we selected seven for detailed study of sex interest and activity which is known to be somewhat defective at this time. On five occasions, actually each alternate night, they were placed with females proven to be in active estrum.[8] In addition to observations during the ensuing hour, the sexes were left together for the night and evidence of sexual union sought the next morning in microscopic search for sperm in the vaginal content. Three of the seven males tested in this way were so defective as to have a record of only one copulation after 15 tests. We now made daily implantations of very potent anterior hypophyseal substance[9] into the leg musculature of these inactive males. The alternate night observations were continued during the implantation period there being eight such tests for each individual after the implantations began. In the 21 tests thus made 12 copulations resulted, an outcome which could not have been expected without treatment. The remaining four old males of the group of eight were tested with care over the same period of time. A total of 40 tests was thus made with them but four copulations resulting. At the conclusion of the tests both the implanted and control groups of animals were sacrificed and careful weighings were made of the testes and accessory organs. A clear hyper-

[8] Such estrous females were selected as follows: Considerable numbers (22 to 40) of females showing the proestrous or early estrous vaginal smear were picked out of a large colony of healthy females. One or two of them in evident outspoken estrum as shown by the usual signs of darting, quivering of ears, etc., were offered to a series of normal males until males with marked sex interest and activity were in this way disclosed. Such males were in turn employed to test the degree of estrous state of the females which had been selected by simply examining the vaginal smear. In this way it was certain that the test females used in all our experiments were not only in the appropriate phase of the vaginal cycle but were individually in active estrum at the time of the test.

[9] Each animal received the hypophyses of three castrated males daily during the first seven days and four daily during the last two days so that each animal received a total of 29 implants. The potency of these implants can be judged by the following: One half hypophysis of a castrate male implanted into the leg musculature of a 24 day old female rat would mature said rat in 98 hours and cause development of ovaries weighing 98 mgm.

trophy of the accessory organs of reproduction resulted from the 11 days of treatment in these old animals.

The sub-joined table (table 12) is sufficiently detailed to serve as a protocol of the experiments, and shows clearly that implantation of anterior hypophyseal substance into senile males (rat) causes a definite increase in sex activity and increase in the weight of the accessory organs of reproduction.

VII. STIMULATING EFFECT OF HYPOPHYSEAL IMPLANTS IN MALE RATS WITH INADEQUATE VITAMIN G. As will be reported elsewhere, the dwarfism produced by entirely withdrawing or lowering the amount of vitamin G is associated with apparent loss of sex function. In the male rat histological studies have surprised us by disclosing an approximately normal condition of the testis and motile sperm in the epididymis, effects in harmony with the capacity of these animals to sire normal litters on the very rare occasions on which copulation was performed. But the overwhelming majority of such animals displayed no sex interest when repeatedly mated with oestrous females. We were interested in discovering whether the administration of hypophyseal substance without change of diets would markedly alter this peculiarity of behavior. The experiments here reported were done with certain individuals from a group of 48 males reared and maintained on diets[10] deficient in the heat-stable water-soluble vitamin G present in yeast. A group consisting of 12 of these males most seriously defective in their sexual physiology was selected by a 60 to 90 day preliminary study period in which each individual was mated on at least six occasions with an oestrous female. The sexual behavior of six of these twelve males was again studied over a period of ten days, during which interval, five one-hour observations were made every other night. After the hourly observation period, males and females were left together for the night, search being instituted the next morning for possible remnants of the copulation plug or for residual sperm in the vaginal canal. In only two instances out of 32 such matings did sexual congress take place. At this point, and for the next 11 days,

[10] The animals were six months of age at the beginning of the experiments described in this paper. The diets were:

Diet 540		*Diet 599*	
Casein (washed and extracted)	25.0	Casein (washed and extracted)	35.0
Sugar	75.0	Sugar	46.0
Salt (no. 185)	4.0	Lard	25.0
		Salt (no. 185)	4.0

Six of the animals (first six animals in table) received no. 599, the other four diet no. 540. Both diets were supplemented by two drops of cod liver oil (adequate for A and D), 100 mgm. autoclaved Fleischmann's yeast (containing vitamin G, but in inadequate amounts), 1 cc. alcoholic extract of rice polishings (adequate for heat labile B), and six drops of wheat germ oil (adequate for E). (B3175 and W2260 received 20 drops of wheat germ oil.)

implantations of hypophyseal substance were made into the leg musculature. The hypophyseal substance used consisted of either one-half or a whole anterior lobe of the hypophysis of adult male rats which had been maintained throughout life on complete diets, and, in addition, had been castrated[11] two or three months preceding the experiment. From the beginning of the implantations, tests of sex activity were made on alternate days, a total of seven such tests being given each individual. In the 42 tests thus made 22 "positive" results or copulations took place.

TABLE 12

Effect of hypophyseal implants on sex organs and reproductive activity of senile male rats

GROUP	DESIGNATION OF ANIMAL	SEXUAL ACTIVITY*				AUTOPSY FINDINGS		
		Preliminary period 11 days		Experimental period 11-17 days		Body weight	Testis	Accessory organs†
		Estrous females offered	Resulting copulations	Estrous females offered	Resulting copulations			
						grams	*grams*	*grams*
Implants	W9959	5	0	8	7**	370	3.90	5.70
	W9960	5	0	8	4**	274	3.20	3.85
	W9967	5	1	5	1**	290	2.55	4.53
Controls	W9951	5	2	5	1	350	3.50	3.50
	W9985	5	1	5	0	260	2.35	2.35
	W1027	5	0	5	0	324	3.10	2.55
	W9906	5	0	5	0	280	2.42	1.70

* When copulations did occur they were fertile.

** Last test period or periods.

† Accessory organs weighed: bladder, penis, Cowper's gland, muscle, prostate, seminal vesicles, coagulating gland.

Those animals which did not copulate every test period invariably copulated in the last three or four of the seven matings. Table 13 is sufficiently detailed to serve as a protocol for the experiments.

At the end of the period of observations the implanted animals and their controls were sacrificed and careful weighings of the testes and accessory organs of reproduction made. The weighings showed clear increases had resulted from this relatively short period of hypophyseal treatment.

To summarize: When the anterior hypophyseal sex hormones are administered to animals dwarfed by inadequate vitamin G and displaying

[11] We have previously shown the marked increase in the gonad-stimulating hormone in the hypophyses of castrated animals when compared with their normal controls, a contrast further exaggerated in the male sex as compared with the female. These hypophyses of castrated males were capable of producing precocious maturity within 100 hours in a 24 day old female rat at a total dose of ½ hypophysis.

little or no sex interest, a marked increase in sex activity (without dietary change) occurs. A change was noted in a week. By the end of the second week normal sex activity was in most cases demonstrable. Distinct hypertrophy of the organs of reproduction was evident in all instances.

VIII. THE NECESSARY CONCURRENCE OF THE OVARY IN MAMMARY HYPERTROPHY IN THE RAT DUE TO HYPOPHYSEAL HORMONES. During the

TABLE 13

Repair of sex deficiencies in male rats maintained on diets deficient in vitamin G by implants of anterior hypophysis

GROUP	DESIGNATION OF ANIMAL	SEXUAL ACTIVITY							AUTOPSY FINDINGS		
		Preliminary period 3 months		Preliminary period 10 days		Experimental period 11 days		Body weight	Testis	Accessory organs	Sperm† motility
		Estrous females offered	Resulting copulations	Estrous females offered	Resulting copulations	Estrous females offered	Resulting copulations				
								grams	*grams*	*grams*	
Implant.....	G2263	7	0	5	0	7	3**	186	1.41	3.50	+ feeble
Control*.....	B3213	6	0			1	1	170	1.25	2.05	+
Implant.....	W2325	5	0	4	1	7	7	205	1.30	4.05	+
Control.....	BH3178	6	0			1	0	208	1.40	2.40	+
Implant.....	B3058	6	0	4	1	7	7	201	1.52	3.75	+
Control.....	B2468	4	2			1	0	210	1.26	3.25	
Implant.....	B2457	5	0	4	0	7	3**	277	1.50	3.06	+ feeble
Control.....	GH2259	7	0			3	0	217	1.26	2.56	+
Implant.....	B3175	6	0	4	0	7	5**	198	1.45	4.05	+
Control.....	W2260	4	1			1	0	204	1.13	1.80	+

* The ten experimental animals were selected from a total of 48 animals on the basis of the poorest mating history. Distribution into experimental and control groups was made on the basis of diet and body weight.

** Last test period or periods.

† Epididymis smears.

last few years considerable attention has been paid in this laboratory to the influence of hypophyseal hormones on the mammary gland. In all cases in which normal females were treated for three or four weeks with aqueous alkaline extracts of the anterior hypophysis rich in the growth hormone, the mammary gland responded by marked proliferation.[12]

[12] Evans, H. M. and M. E. Simpson. Hyperplasia of mammary apparatus of adult virginal females induced by anterior hypophyseal hormones. Proc. Soc. Exp. Biol. and Med., 1929, xxvi, 598.

The effect of such hypophyseal extracts was always greatly emphasized by more chronic administration. So striking was the phenomenon in chronically injected virginal rats that in some cases milk exuded from the mammary gland when the latter was incised at autopsy. The mammary development resembled closely that at the height of lactation.

Another even more striking phenomenon was encountered in females injected with such extracts for 12 to 18 months in an effort to produce maximal gigantism. We refer to the formation of multiple mammary tumors. Localized enlargements in the mammary line began to appear in animals of 15 months of age which had been injected for 11 months. The swellings appeared more frequently in the axillary or groin mammae but were found wherever mammae occur. When these enlargements first appeared they were soft and lobulated. As the tumors enlarged they became progressively firmer. Some of the larger tumors were so tough they could scarcely be cut with a sharp scalpel. They were grey white in color and limited in outline. Frozen sections stained with sudan III and hematoxylin were examined by Dr. G. Y. Rusk of the Department of Pathology of the University of California and the diagnosis of adenofibroma established.

Epithelial cells arranged around a lumen could easily be discerned in the softer, younger tumors. In the firmer tumors the epithelial cells were almost completely obliterated by dense fibrous tissue. Sixty-two per cent (10 out of 16) of the animals which lived to be 16 months of age receiving daily intraperitoneal injections of 17A developed one to six lobulated tumors. Some of the larger tumors reached very great dimensions. In one animal whose total weight was 700 grams the tumor reached a weight of 263 grams. None of the tumors metastasized. The tumor formation observed in these cases may be related to the greater frequency of mammary tumors in women in whom ovarian dysfunction has for years produced menstrual irregularities.

More marked mammary responses can be produced in adult virgin rats than those described above as the result of injecting hypophyseal extracts rich in the growth hormone. Striking reactions occur after treatment with implants of rat hypophyses or with extracts of human placenta and the urine of pregnant women. The hyperplasia is remarkable after but ten days' dosage by the latter methods.

In infant rats, also, that have been subjected to treatment with products inducing precocious sexual maturity, the mammary apparatus undergoes hyperplasia.[13] In mammary studies with infant rats we usually employed females 24 days of age and sacrificed them after six days. Every

[13] Evans, H. M. and M. E. Simpson. Hyperplasia of mammary apparatus in precocious maturity induced by anterior hypophyseal hormones. Proc. Soc. Exp. Biol. and Med., 1929, xxvi, 598.

method known to us at the time for promoting precocious maturity, caused a definite increase in the complexity of the mammary tree within this interval, (e.g., rat and bovine implants, aqueous acid extracts of placenta and urine of pregnant women precipitated by alcohol—the latter two products being practically free of folliculin and growth hormone). The effect of implants in developing the mammae of immature rats was so prompt that changes were found after but two daily implants followed by sacrifice on the fourth day.

The possibility that some hypophyseal hormones might induce merely development of ovarian follicles and that others might induce an overgrowth of lutein tissue led us to hope that in this way we could test the dependence of mammary hyperplasia on the existence of lutein tissue in the infantile ovary. Our treatments unfortunately brought about the development of both follicles and corpora in the young ovaries. But clear instances were found of the beginning of mammary hyperplasia before lutein tissue existed in the ovary, even though all cases of maximal development of the mammary tree coexisted with the presence of both corpora and follicles. We concluded, therefore, that the secretion of corpora lutea could not have been responsible for the initial stimulus to mammary growth.

The absence of mammary hyperplasia in chronically treated males[14] and the entire absence of mammary tumors in this group diverted us from the attempt to produce mammary responses in gonadectomized females. In the rat the data had impressed us as indicating the necessity of ovarian mediation in these striking effects. It had, moreover, been established by Edgar Allen and others that one could obtain certain degrees of mammary hyperplasia with the ovarian follicular hormone. Recently Corner[15] has tested the effect of his lutein hormone "progestin" on the mammary gland of spayed non-pregnant female rabbits and was surprised to find it inefficacious. On the other hand he secured mammary hyperplasia and active lactation by injection of an extract of sheep's hypophysis into virginal spayed rabbits. The presence of the ovary did not increase or alter the extent of the reaction produced. Corner says in conclusion "these experiments demonstrate the production by extract of sheep's hypophysis of changes in the mammary glands of rabbits resembling those occurring in pregnancy, and leading to secretion of milk. The effect does not depend on the ovaries, for it can be produced in animals spayed at the beginning of the experiment. Very likely, however, the mammary glands must first be subjected to the influence of oestrin (ovarian follicle hormone) and thus be brought into full pubertal condition at the time of spaying and the beginning of the injections."

[14] A large group of males was treated for 1½ years with 17A and another alkaline solution (18) richer in both protein and hormone.

[15] Corner, G. W. Hormonal control of lactation. This Journal, 1930, xcv, 43.

Although Corner contented himself with the clear statement of these remarkable results in the rabbit, the title of his paper, "The Hormonal Control of Lactation," and its subtitles, "Non-effect of the corpus luteum" and "Positive action of extracts of the hypophysis," might lead one to infer that generalization was now possible. We are beginning to realize that marked differences exist among the mammalia in the various steps or phases of hormone function connected with reproduction. One can not detect in the cow or rat the gonad-stimulating hormones characteristic of the blood and urine of pregnant women and mares. Hisaw reports the presence of his lutein hormone in the blood-serum of pregnant rabbits, sows, dogs, cats and mares but its absence in the serum of pregnant cows and women. The folliculin content of serum also varies in different animal forms.

Mammary hyperplasia is notoriously easy to produce in rabbits. It is much more difficult to provoke in some other forms, for example, in the rat. Yet under appropriate conditions the mammary gland of the rat hypertrophies maximally. For these reasons we have re-examined the question of mammary response to the anterior pituitary in the rat. We injected spayed virgin rats with alkaline extract of beef anterior hypophysis for periods of 20 to 30 days.[16] We also subjected the test females to the most powerful mammary stimulant we know, namely, implants of rat hypophyses. We planned these experiments so we could compare the response in the mammary trees of treated spayed animals with those of normal litter mate sisters treated in the same way. Mammary glands of untreated normal and spayed sisters were used as controls. (See table 14.) Care was taken to use three types of spayed females, those spayed three days and those spayed one month and eleven months respectively before the experiment, for it was thought that internal secretions of the ovary involved in mammary development might continue to circulate for a short time but be absent in the animals castrated a longer interval. The results of this work were the same, however, in all cases; regardless of the time of castration, the mammary gland of the castrate did not respond, as can be seen in table 15. In the rat a great and decisive difference is made whether the ovary is present or not. Any considerable degree of hypertrophy or approach to actual function of the mammary gland which was produced

[16] Mammary development was studied in this series of experiments 20 to 30 days after onset of treatment because experience with normal adult female rats had previously shown us that this length of injection with our standard alkaline extract of the hypophysis (17A) was necessary to provoke marked development. There appeared to be no objection to this duration of treatment with other hypophyseal substances as we have observed no evidence of regression of mammary development in this period of treatment with more potent hypophyseal mammary stimulants, e.g., hypophysis implants. Implants provoke marked hypertrophy in the gland within 10 days but the development is even greater after 30 days' treatment.

TABLE 14

Effect of anterior hypophysis administration on the mammary gland of ovariotomized rats

NORMAL						OVARIOTOMIZED*				
Experiment	Designation of animal	Length of treatment	Gain in body weight 20 days	Weight of ovary at autopsy	Mammary§ development at end of treatment	Experiment	Designation of animal	Length of treatment	Gain in body weight 20 days	Mammary development at end of treatment
		days	*grams*	*mgm.*				*days*	*grams*	
Control to 17A injected and hypophysis implanted	GH2661		11	75	+	Control to 17A injected and hypophysis implanted	B2711		10	0
	BH2709		10		0		B2755		14	0
	W2750		4	60	0		B2665		4	0
	B2754		−16		0		B2685		8	0
	B2756		−2	86	0		W2695		4	0
	B2684		−2		0		BH2698		0	0
17A**	B2688	30	48	101	+++	17A	G2751	20	42	+
	BH2699	20	52	140	+++		W2694	20	70	+
	B2749	20	44		++		GH2761	20	22	0
	BH2663	20	58	80	++		BH2671	30	14	0
	B2757	20	50	115	+		GH2675	30	38	0
	GH2676	30	34	31	0		BH2687	20	34	0
Hypophysis† implants	B2758	20	−10	450	+++++	Hypophysis implants	B2662	20	16	0
	BH2673	30	8	1,210	+++++		B2674	30	−4	0
	W2696	20	28	875	+++++		G2678	20	−12	0
	B2689	30	28	950	++++		BH2686	30	0	0
	G2677	20	6	510	++++		GH2752	20	1	0
	BH2660	20	−2	170	++++		W2693	20	−2	0
Control to antuitrin	BH2800		14		+	Control to antuitrin	G2776		24	0
	BH2768		6	65	0		GH2785		18	0
	GH2775		2	65	0		BH2790		32	0
	B2791		8	35	0		GH2771		18	0
	BH2787		−16		0		W2798		32	0
	W2805		6		0					

Antuitrin G‡	BH2769	20	32	70	++	Antuitrin G	W2765	20	52	+
	G2784	20	40	55	++		GH2774	20	44	+
	BH2789	20	50	130	++		B2797	20	65	+
	B2792	20	30	90	++		BH2777	20	33	0
	GH2772	20	8	90	+		G2783	20	47	0
	B2806	20	50	110	0		B2788	20		0

* The animals used in these experiments were 5 to 6 months of age. Those in the first three series were spayed one month before the experiment: in the last two series, i.e., the antuitrin experiment, four days before.

** The standardized alkaline extract of bovine anterior hypophysis (17A) promotes an average growth in adult female rats of 52 grams per 20 days. It also inhibits oestrous cycles. The dose given here was 2 cc. daily intraperitoneally.

† These animals received intramuscularly on alternate days one half hypophysis from a castrated male rat. The donors had been castrated 11 to 19 months. Each hypophysis was potent enough to precociously mature a 24 day old female rat in 100 hours with the stimulation of 96 mgm. ovaries.

‡ Antuitrin G was kindly supplied us by Parke, Davis & Co. for comparison with our own alkaline extracts of bovine hypophysis. Dose 1 cc. 10 days, 1½ cc. last 10 days.

§ The cleared stained mammary spreads were studied in oil of wintergreen under a dissecting binocular. The degree of development has been expressed as follows: 0, no response, naked ducts; + barely detectable response; ++ definite early response; +++ considerable response; ++++ marked response; +++++ maximal response.

by hypophyseal therapy was dependent on the concurrent presence of the ovary. (See also fig. 1.)

All the studies cited above were made, however, with virgin rats. The possibility therefore existed that the refractory state of the mammary tree in virgins was due to the fact that it had never been subjected to the secretion from a functional corpus luteum. (This assumes that the corpus luteum of the normal rat cycle is not functional and only becomes functional when its life is prolonged by normal copulation, copulation with

TABLE 15

Effect of time of castration on the response of the mammary tree of the rat to anterior hypophysis extracts

SPAYED FEMALE CONTROLS				SPAYED FEMALES INJECTED WITH 17A**			
Time spayed* before experiment began	Designation of animal	Age	Mammary† development 30 days after beginning of experiment	Time spayed before experiment began	Designation of animal	Age	Mammary development after 30 days of treatment
		months				*months*	
4 days	W3659	4	+	4 days	BH3690	4	++
	W3360	4	0		W3358	4	+
	B3677	4	0		BH3665	4	+
	BH3686	4	0		B3678	4	+
	BH3689	4	0		W3658	4	0
					BH3687	4	0
11 months	W4095	15	+	11 months	BH3631	15½	+
	W3624	15½	0		W3640	15	+
	W3630	15½	0		B4071	15	+
	BH3639	15	0		BH4093	15	+
	B4070	15	0		GH3621	15½	0
	G3619	15½	0		B3627	15½	0

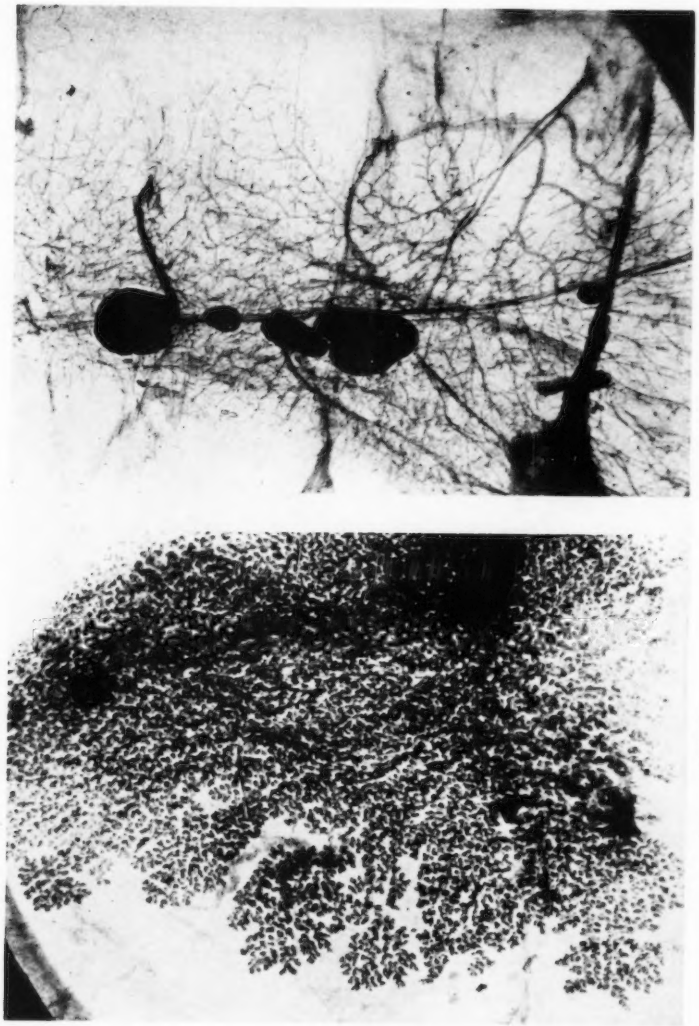
* See also table 14 in which animals are listed in which the experimental period occurred from four days to one month after castration.

** Dose 2 cc. daily intraperitoneally.

† The degree of mammary development is graded as in table 14.

a vasectomized male, glass rod stimulation of the cervix, or by hypophyseal extract administration; function of the corpus luteum being measured by ability of the uterus to respond by nidation of eggs, or production of placentomata about a silk thread, and in the vagina by the production of the characteristic pregnancy or pseudo-pregnancy epithelium.)

The following experiment put this hypothesis to the test. Fifty-four animals were bred during the same night. These animals were divided into six groups of nine each. Two groups (see table 16) were spayed approximately 18 hours after mating. Each animal in the first of these groups received four rat hypophyses as implants on alternate days for a period



Ovariotomized BH2686
× 2½

Normal W2696
× 2½

Fig. 1. Effect of anterior hypophysis administration on the mammary gland of normal and ovariotomized rats (table 14).

TABLE 16

Effect of anterior hypophysis administration on the mammary development of rats ovariotomized after copulation

CONTROLS						HYPOPHYSIS IMPLANTS*					
Experiment	Designation of animal**	Gain in body weight 10 days	Ovary weight at autopsy	Implantation sites	Mammary† development 10 days after copulation	Experiment	Designation of animal	Gain in body weight 10 days	Ovary weight at autopsy	Implantation sites	Mammary development 10 days after copulation
		grams	*grams*					*grams*	*grams*		
Spayed 18 hours after copulation	BH9113	−6		0	+	Spayed 18 hours after copulation	W9229	18		0	+
	BH9220	12		0	+		W1300	4		0	+
	G1429	30		0	+		W9112	32		0	+
	BH9463	9		0	+		W9167	6		0	+
	BH9121	22		0	0		BH9234	20		0	0
	W9232	28		0	0		B9140	20		0	0
	W1098	4		0	0		B4748	24		0	0
	B1091	20		0	0		BH4754	26		0	0
							BH9596	7		0	0
Spayed 3½ days after copulation	BH1306	14		0	+	Spayed 3½ days after copulation	BH1302	−6		0	+
	BH1377	6		0	+		W9152	−5		0	+
	BH1294	3		0	+		BH1393	10		0	+
	BH4737	6		0	0		B9204	0		0	+
	BH4738	−9		0	0		B1055	−6		0	+
	BH1389	8		0	0		BH1387	−4		0	+
	BH1391	−6		0	0		B1400	4		0	0
	GH1293	14		0	0		B9205	13		0	0
	BH1297	−0		0	0		B1440	0		0	0

Pregnant	GH1428	42	88	Normal	++++	Pregnant	B9603	13	92	Normal	++++
	W1395	20	81	Normal	++++		G9115	−43	125	Resorbing	++++
	W9122	16	70	Normal	+++		W1906	−4	85	Resorbing	+++
	BH1375	20	70	Normal	+++		BH9123	22	103	Resorbing	+++
	GH4725	26	62	Normal	++		B9130	10	81	0‡	+++
	W1431	12	61	Normal	++		W9465	0	84	Resorbing	+++
	B1441	16	64	Normal	++		W4752	−2	191	Resorbing	+++
	BH1391	4	91	Normal	++		W9457	5	107	Normal	+++
	BH1388	13	45	0‡	+		BH9570	−4	88	0‡	+++

* Each animal received intramuscular implants of four anterior hypophyses of adult rats (3 female and 1 male) on alternate days during the 10 day period, i.e., in five implantations each rat received a total of 20 anterior lobes.

** All animals in this experiment were 8–9 months of age.

† The degree of mammary development is graded as in table 14.

‡ The uterus was very large and flaccid.

of ten days. The other group acted as control. Two other groups were not spayed until 3½ days after copulation. The first of these groups received implants on alternate days; the latter group acted as control. The remainder of the animals served as two further types of controls, namely, pregnant-implanted and normal-pregnant controls.

All animals were autopsied on the 10th day after copulation. As can be seen in the table the results were the same as those obtained with virgin animals which had never been subjected to hormones freed after copulation. Even where normal pregnancy had been allowed to continue for 3½ days before castration, the mammary gland consisted only of naked ducts. This was equally evident in the animals receiving the powerful stimulation of the implant and in the untreated animals spayed at the same time. On the other hand the mammary glands of the control animals with ovaries reacted màximally to the stimulus of the hypophysis implants. Some indication of the magnitude of the stimulus is given by the weights of the ovaries of these controls. (See table 16.)

In conclusion we can only reiterate that the rat mammary gland shows practically no response to hypophyseal administration unless the ovary is present. Future research may identify this necessary participation of the ovary with one or more of the known hormones of the ovary.

SUMMARY

1. For assay of the anterior hypophyseal growth hormone six "plateaued" female rats from five to eleven months of age are employed. In each individual case, growth cessation is proven by at least three weighings at five day intervals. The "plateau" is defined as a maximal gain of ten grams in twenty days. 2. Male rats are more variable than females in their capacity to respond to the growth hormone. 3. Under the influence of the growth hormone, male rats are incapable of adding to their body substance to the degree which characterizes females. This is still the case after gonadectomy of both sexes so that male and female tissues react differently to the growth hormone. 4. The growth as contrasted with that of their controls is exhibited best by injected gonadectomized females and least by injected normal males. Normal males are, however, always the greatest giants produced by chronic administration of the growth hormone. 5. Animals suffer an actual loss of weight for from ten to fifteen days after administration of the growth hormone is stopped; their body weight is however permanently greater than that exhibited by their uninjected controls. Cessation of treatment entails no recession in the increased skeletal dimensions produced by the growth hormone. 6. Sporadic congenital dwarfism is readily amenable to alteration by administration of the growth hormone. 7. Implantation of anterior hypophyseal substance into senile male rats causes a definite increase in sex activity and increase in weight of the accessory organs of reproduction. 8. Implantation of anterior hypophyseal substance into male rats rendered dwarfed and subnormal in their sexual physiology by inadequate vitamin G—increases markedly their sex activity, establishing normal behavior in fact within a fortnight. 9. Implants or extracts of anterior hypophyseal substance cause marked hypertrophy of the mammary gland in the rat when the ovary is present but they are incapable of doing so after ovariotomy.